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DEDICATION

To My Mother,
For Her Love and
Confidence Throughout
the Years

ABSTRACT

The hepatic cytosolic system has been used to study the process of mammalian carbonyl reduction.

In vitro metabolism of the aromatic ketone propiophenone and its non-aromatic isomer phenylacetone, as well as several analogs of both compounds, were extensively investigated using fortified liver preparations [10 000X*g* supernatant, 105 000X*g* microsomal fraction, 105 000X*g* cytosol (soluble) fraction] from rat and rabbit. Reduction to the corresponding alcohols was the major pathway observed, although aliphatic *C*-hydroxylation also occurred, as did dehydrogenation of the product alcohols. Metabolites were identified and quantitated by gas liquid chromatography (GLC) and GLC/mass spectrometry (MS), and these analytical procedures are discussed. The nature and quantities of metabolites showed both species and cofactor dependencies.

Methodologies employed in the *in vitro* experimentation are discussed in detail. Conditions were investigated which would permit maximum enzyme activities of both carbonyl reduction and aliphatic hydroxylation. The metabolic reductase system could be defined further with regard to pH optimum, NADH-synergism, subcellular location and substrate specificity.

The stereospecificity of the metabolic reduction of arylakylketones was studied. Ketones propiophenone, phenylacetone, and 1-phenyl-1,2-propanedione were reduced *in vivo* and *in vitro* in rat and rabbit to the corresponding alcohols. For the analysis a capillary-GLC method employing chiral derivatizing reagents for the resolution of these optically active alcohols was utilized.

The metabolism of amphetamine and related compounds by rat brain tissue was investigated _in vivo_ and _in vitro_.

Conditions were studied for the extraction, separation and quantitation of amphetamine and norephedrine, and their amphoteric metabolites _p_-hydroxyamphetamine and _p_-hydroxynorephedrine, in biological fluids, using electron-capture GLC. The procedure utilizes the ion-pairing reagent, di(2-ethylhexyl)phosphoric acid, the use of which separates the amines from most endogenous contaminants. The assay procedure further permits the efficient extraction of the amphoteric metabolites following their conversion, in aqueous medium, to acetate derivatives. Amines amphetamine and norephedrine, and the acetylated derivatives of the amphoteric compounds are perfluoroacylated prior to EC-GLC analysis. Mass spectra were obtained for the acetylated and/or perfluoroacylated derivatives. The sensitivity and specificity of this method allows the quantitation of these metabolites down to 10 ng/g tissue when present in the brain.

Rat brain metabolism of amphetamine, _p_-hydroxyamphetamine, and norephedrine was investigated. The data was interpretated as showing that both _in vivo_ and _in vitro_, amphetamine and norephedrine are _para_-hydroxylated to _p_-hydroxyamphetamine and _p_-hydroxynorephedrine, respectively, and _p_-hydroxyamphetamine was _beta_-hydroxylated to _p_-hydroxynorephedrine. Norephedrine was not detected as a rat brain metabolite of amphetamine. All results were confirmed by selected ion monitoring utilizing chemical-ionization MS.

Rat hepatocytes in suspension and monolayer primary cultures were evaluated as _in vitro_ model systems for the investigation of the metabolism of foreign compounds.

Parameters conditioning isolation of viable cells, attachment efficiency, and optimum survival of rat liver parenchymal cells were defined for culture systems. More than 2.5 x 10^8 viable mature cells were routinely isolated by collagenase perfusion of adult rat liver. Culture conditions included the use of an enriched media mixture (DMEM/F-12), fetal bovine serum, and supplementation with insulin and dexamethasone.

Comparative morphological and biochemical studies of adult rat hepatocytes cultured on glass, plastic, thin collagen layer, collagen gel and floating collagen membrane, and of cells entrapped within a semi-permeable carrageenan matrix are described. Results are discussed in relation to the necessity of cultured cells establishing a proper socio-cellular architecture, reminiscent of hepatic tissue *in vivo*, in order to remain viable *in vitro*. Rat liver cell cultures established from fetal/neonatal tissue were found to possess few characteristics of mature hepatic tissue.

The preparation of adult rat hepatocytes provided a high yield of intact metabolically active cells. The hydroxylation of amphetamine to *p*-hydroxyamphetamine was investigated in several *in vitro* culture systems and compared to that found *in vivo* and with broken cell liver preparations. These results support the validity of adult hepatocytes in short term primary monolayer cultures as a model system for qualitative investigations of the metabolic fate of xenobiotics in mammalian cells.

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LIST OF ABBREVIATIONS

α	alpha
AA	acetic anhydride
ADH	alcohol dehydrogenase
AlR	aldehyde reductase
β	beta
CI	chemical ionization
CG	collagen gel
d.	day(s)
d	dextrorotatory
D	deuterium (deuterated)
DEHPA	di(2-ethylhexyl)phosphoric acid
DMEM	Dulbecco's Modified Eagle Medium
EC	electron capture (detector,detection)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-*bis*(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid
EI	electron impact
FBS	fetal bovine serum
FCM	floating collagen membrane
FID	flame ionization detector (detection)
GLC	gas liquid chromatography (chromatographic)
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
ic.	intracisternally
ip.	intraperitoneally
IR	infrared
ISCDH	isocitrate dehydrogenase
l	levorotatory
LADH	liver alcohol dehydrogenase
LDH	lactate dehydrogenase
MBIC	*S*-(+)-α-methylbenzylisocyanate
MCF	*R*-(-)-menthyl chloroformate
MEM	Minimum Essential Medium
MFO	mixed function oxidase
MS	mass spectrometer (spectrometry)

n	nano (10^{-9})
NAD	nicotinamide adenine dinucleotide
NAD+	" " " oxidized form
NADH	" " " reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADP+	" " " " oxidized form
NADPH	" " " " reduced form
NMR	nuclear magnetic resonance
p	para
p	pico (10^{-12})
PBS	Phosphate Buffered Saline
p.c.	post conception
PFP	pentafluoropropionyl
PFPA	pentafluoropropionic anhydride
psi.	pounds per square inch
R	rectus
S	sinister
SCOT	support column open tubular
SIM	selected ion monitoring
TAT	tyrosine aminotransferase
TCL	thin collagen layer
TFA	trifluoroacetyl
TFAA	trifluoroacetic anhydride
TIC	total ion current
TPB	tetraphenylboron
u	micro (10^{-6})
U	unit(s)
UV	ultraviolet

DEFINITIONS

Anchorage-Dependent Cells or Cultures. Cells, or cultures derived from them, which will grow, survive, or maintain functions only when attached to a surface. (The use of this term does not imply that the cells are normal or that they are not malignantly transformed.)

Attachment or Seeding Efficiency. That percentage of the inoculum which attaches to the surface of the culture vessel within a given period of time.

Cell Line. A cell line arises from a primary culture at the time of the first subculture. An established cell line is a cell line which demonstrates the potential to be subcultured indefinitely *in vitro*.

Cell Strain. A cell strain is derived either from a primary culture or a cell line by the selection or cloning of cells having specific properties or markers. These properties or markers must persist during subsequent cultivation.

Chemically Defined Medium. A nutritive solution for culturing cells in which each component of the medium is of known chemical structure.

Culture:

cell culture - used to denote the maintenance or growth of cells *in vitro*, including the culture of single cells. In cell cultures, the cells are no longer organized into tissues.

diploid culture - a culture having, arbitrarily, at least 75% of the cells having the same karyotype as the normal cells of the species from which the cells were originally obtained.

explant culture - excised fragment of a tissue or organ which is transferred to an artificial medium for growth.

heteroploid culture - a culture having less than 75% of cells with a normal diploid chromosome constitution.

monolayer culture - refers to a single layer of cells growing on a surface.

organ culture - the maintenance or growth of tissues, organ primordia, or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function.

primary culture - implies a culture started from cells, tissues, or organs taken directly from the organism. A primary culture is regarded as such until it is subcultured for the first time, when it becomes a cell line.

suspension culture - denotes a type of culture in which cells are grown while suspended in medium.

tissue culture - the maintenance of tissue fragments *in vitro*, not necessarily in conditions designed to preserve tissue architecture.

Dedifferentiation or Fetalization The loss, by highly differentiated cells, of characteristics/functions specific to their mature state. The cells essentially return to an infantile-like stage of development.

Differentiation. The diversification process of cells acquiring completely individual characteristics specific for certain functions.

Epithelial Cells (Epithelial-like Cells). Resembling or characteristic of, having the form or appearance of epithelial cells. In order to define a cell as an epithelial cell, there must be definitive characteristics present typical of epithial cells; i.e., appear cuboidal when viewed under the light microscope and grow in continuous mosaic-like sheets with very little intercellular substance, wherein cells are in quite close contact with each other. Usually one is certain of the histological origin and/or function of the cells placed into culture and, under these conditions, one can be reasonably certain in designating the cells as epithelial.

Fibroblasts (Fibroblast-like Cells). Resembling or characteristic of, having the form or appearance of fibroblast cells. In order to define a cell as a fibroblast cell, there must be definitive characteristics present typical of fibroblast cells; ie, appear pointed or elongated when viewed under the light microscope and grow in sheets wherein the cells are rather loosely in contact with one another.

Mitosis. The process by which the body replaces cells. A method of indirect division of a cell, consisting of a complex of various processes, by means of which the two daughter nuclei receive identical complements of the number of chromosomes characteristic of the somatic cells of the species.

Morphogenesis. The evolution and development of form, as the development of the shape of a particular cell or organ.

Neoplastic. A description referring to a new or abnormal growth or proliferation of cells. Usually refers to an abnormal cancerous proliferation with the ability to produce tumors in appropriate hosts.

Ploidy. The number of chromosomes sets. Haploid - the basic chromosome number of a polyploid series, in this instance meaning monoploid. Diploid, Triploid, Tetraploid, etc - double, triple, quadruple, etc. the basic number. Polyploid - general designation for multiples of the basic number, higher than diploid.

Subculture. The transfer or transplantation of cells from one culture vessel to another. This term is synonomous with the term "passage".

Transformation (In Vitro). A heritable change, occurring in cells in culture. This change occurs intrinsically or results from treatment with chemical carcinogens, oncogenic viruses, irradiation, etc., and leads to the acquisition of altered properties such as morphological, antigenic, proliferative, etc. Different from neoplastic alterations in that it does not include the ability to produce cancerous tumors *in vivo*.

1. INTRODUCTION

'Patients may recover in spite
of drugs, or because of them'

J.H. Gaddum, 1959

The introduction of a drug into a living system may not always result in the expected effect. As drugs, or any foreign compounds, are chemical in nature, any response they initiate can usually be interpreted as the consequence of chemical reactions occurring at the subcellular level. Such interactions, which can occur with a wide variety of endogenous components, evolve as biochemical, physiological, or behavioral changes. But as important as it is to assess how a drug (or any chemical form) influences a biological system, it is equally as important to examine just what effect the system may have on the drug. This discipline of pharmacology, generally referred to as drug or xenobiotic metabolism has substantially improved the fundamental understanding of drug-body interactions. Metabolism, originally considered to be merely part of the drug elimination process, was inaccurately referred to as a simple mechanism for detoxification. It soon became evident however, that the metabolism of an active compound was not always accompanied by a loss of its pharmacological effect. Indeed, after many observations that various biologically active substances actually lacked their efficacy per se and only become pharmacologically active upon biotransformation, or that certain compounds could give rise to toxic, mutagenic, or carcinogenic products, it was eventually recognized that metabolism no longer necessarily implied only detoxification or inactivation. As a result, this once simple concept gave way to a much more complex scheme. The overall effect of a drug on a living body depends not only on the compound's physiochemical properties which control the nature of the response, but also upon the physiological and biochemical status of the system, which determines the fate of the drug in that body.

1.1 DRUG METABOLISM STUDIES

The biotransformation of xenobiotics involves a vast multi-component enzyme system through which changes are generated by way of well established metabolic reactions. Although the literature dealing with these pathways is extensive, fundamental knowledge in many areas is at best, fragmentary. Much remains to be learned about the capability of biological systems to alter compounds which are essentially completely foreign in nature to the body, and about the mechanisms involved in the control of the metabolizing enzymes. By understanding the metabolism process more thoroughly, a better awareness of how and why drugs work is provided.

The principal aims of xenobiotic metabolism studies have been outlined by Hathway (1) as the provision of information on

i. the overall adsorption, distribution and excretion of a foreign compound and its metabolites,
ii. the kinetics of absorption, distribution, biotransformation, and excretion,
iii. the identification of metabolic products and on the elucidation of related metabolic pathways,
iv. the enzymic mechanisms responsible for the observed biotransformations, and
v. the fundamental biological parameters which regulate the course of foreign compound metabolism.

1.1.1 In Vivo Studies

The obvious approach by which to investigate drug metabolism and any resulting pharmacological and toxicological consequences is through the utilization of in vivo experimentation. Studies on live animals has made it possible to establish metabolic pathways and determine various factors which affect the levels of drugs and their metabolites. However, this manner of research is subject to several disadvantages:

- *i*. Animals are often exposed to uncontrollable and many times unidentifiable factors which may significantly influence the results (ie. disease, genetic factors, microbial flora, etc.).
- *ii*. The procedure has very little control over such important aspects as the distribution of the drug, its concentration in a specific tissue, and the time of exposure at that site.
- *iii*. While extremely useful in establishing the overall fate of a drug, *in vivo* studies do not present adequate information about the mechanisms of drug biotransformations at the cellular level.

Because of these drawbacks, supporting *in vitro* analyses are sometimes necessary to provide a more satisfactory interpretation of the *in vivo* results. Although only a limited assessment of the general behavior of drugs can be made *in vitro*, these investigations do however provide a more intricate understanding about the actual metabolism process than is possible with *in vivo* studies.

1.1.2 *In Vitro* Studies

Prior to any practical clinical application of a drug, considerable time and effort is devoted to characterizing the drug from a pharmacological and toxicological standpoint. As with *in vivo* studies, *in vitro* investigations are useful in aiding the extrapolation of animal data from one species to another, or more importantly, from animal to man. Gillette

(2) summarized the purposes of in vitro studies as procedures

- i. to determine in which tissue drug transformation takes place,
- ii. to determine the physiological and chemical steps involved in the formation of metabolites and to identify the components of the enzyme systems that mediate these steps,
- iii. to characterize the biochemical properties of the various components which make up the enzyme systems, and
- iv. to determine the various mechanisms and factors which control enzyme activity.

1.1.2.1 Broken Cell Preparations, Tissue Slices and Perfused Organs

Many of the advancements made in elucidating the complex drug metabolism process have been through the use of various forms of in vitro experimental models. By far the majority of investigations have utilized broken cell preparations derived from liver tissue, which include whole tissue homogenates, subcellular fractions, and more recently, purified and partially-purified enzymes. These in vitro models do, however, suffer from certain drawbacks which have lead to a serious point of issue (3). Since the architecture of the native cells may play a significant role in the regulation of metabolism in the intact animal, the assumption that reconstituted enzyme systems, prepared from homogenized tissue, can be used to accurately represent the reaction mechanism of a living animal is of questionable validity. Once the interrelations of a multiple enzyme system are destroyed by homogenization, the sequence of biochemical events may be irreversibly changed. Subsequently, efforts have been made to develop an in vitro model which more closely resembles a true in vivo situation. Because cellular architecture remained essentially unchanged, many promising experimental techniques have involved either tissue slices or perfused organs. Unfortunately, both preparations also have notable disadvantages. With liver slices for instance, metabolism is slow, due in part to the difficulty with which substrates diffuse into the fragments. Furthermore, a large fraction of the cells are inevitably damaged as a result of the slicing procedure and reproducible results are difficult to obtain as cellular necrosis within the center of the slices usually occurs.

For the study of intact liver functions under controlled conditions, perfused livers have been extensively utilized. This experimental system is of considerable value when investigating such specific topics as biliary clearance, blood-flow effects and first-pass kinetics. Unfortunately, these experiments are technically difficult, time-consuming in as much as only one variable can be measured with each organ, of limited viability (4-8 hours), and conditions are difficult to reproduce (4). Because of these difficulties, the procedure has not aquired wide-spread acceptance. Nevertheless, since perfused organs can be maintained under conditions resembling those which occur in the live animal, these in vitro preparations can provide valuable information on the metabolic capabilities of the tissues involved.

1.1.2.2 Isolated Cells and Tissue Cultures

In recent years the development of systems using freshly isolated cells has become increasingly attractive for the investigation of xenobiotic biotransformations. Many of the problems associated with other intact cell models (liver slices, perfused liver) do not arise when isolated hepatocytes maintained as either suspension or monolayer cultures are utilized. The major appeal of isolated hepatocytes is that they provide the simplest model in which drug metabolizing enzymes retain virtually the same properties as in the in vivo situation. This system offers an alternative to the more complex and troublesome studies with isolated perfused livers, but without the loss of cell integrity associated with the use of liver cell homogenates and subcellular fractions. The particular advantages of isolated cells have been summarized by a number of authors (5-8):

i. The viability of the system is much longer than with perfusion or slices. This is particularly true of monolayer cultures.

ii. It is possible to control the culture medium, allowing the surrounding environment to be completely defined.

iii. The systems are essentially free of any major variables, i.e. hormone, nutrient levels, etc.

iv. They allow the study of functions specific to the cell without the difficulties caused by the presence of other unrelated cells in a tissue.

v. There is a much more rigid control of dosing concentrations and time of exposure to the drug than in in vivo studies.

vi. It is possible to conduct direct studies of changes going on within the cell, such as protein synthesis, enzyme levels, etc. This would allow observations to be made of any relationship between metabolism and toxicity.

vii. Repeated sampling from a single batch of cells is possible, so that both intracellular and extracellular measurements can be made simultaneously and in relation to time. As well, it is possible to divide cells derived from the same animal and run paired control and test cultures.

The application of this relatively uncomplicated model, containing both an intact cell membrane and a viable drug-metabolizing system, can be of significant value in determining the direct pharmacological and/or toxicological effects of a compound in a cell, and the metabolic changes mediated by cells on a drug. However, as with any viable experimental system, the approach to using isolated cells also has various drawbacks. In some studies, cells have demonstrated very poor, or no metabolizing ability, and some doubt has been raised concerning the value of isolated hepatocytes in drug metabolism investigations.

Understandably, no single technique used for studying drug metabolism has been able to provide all the answers. This emphasizes the continual necessity for improvement of existing systems and the development of new models for the comprehensive analysis of both the compounds under study and the manner in which a viable biological body behaves.

2. AIMS AND OBJECTIVES

The primary objectives of the present research program involved two major aspects of drug biotransformation investigations. The first area was concerned with the utilization of in vitro animal models during metabolism studies. Significant interest was focussed on improving the understanding about such in vitro experimental systems and their application to current research. The second area was involved with the analytical methodologies used in the determination of xenobiotics and their metabolites. The development of specific and sensitive analytical procedures, especially ones applicable to compounds in biological materials, play a major function in unravelling the complex process of drug metabolism.

A large number of compounds of biological interest are found to possess a ketone functional group. These include medicinally important drugs, as well as a large variety of other xenobiotics to which exposure may occur, such as food additives, agricultural chemicals, or organic solvents. In addition, many non-ketone compounds may be metabolized to ketones, as with the oxidative deamination of amphetamine to phenylacetone. The metabolic fate of xenobiotic carbonyl containing compound is therefore of great interest, and it has been found that reduction to the corresponding alcohol is a significant pathway for the biotransformation of both aldehydes and ketones in mammalian tissue. However, whereas in recent years aldehyde reductases from several species have been isolated and extensively characterized, information on the biological and physical properties of ketone reductases is still lacking. For this reason a detailed study of the metabolic reduction of several appropriate aliphatic and aromatic arylalkylketones was undertaken, including investigations dealing with species, cofactor and substrate differences.

Due to the nature of metabolic reduction, that is, conversion of an achiral ketone to a typically asymmetric alcohol product, an opportunity was further provided to investigate several stereochemical aspects in the metabolism of drugs. Few detailed studies on the stereochemical mechanism of ketone reduction have been published. A major reason for this absence of information has been due, at least in part, to the lack of sufficiently specific and sensitive analytical techniques for the determination of the optical purities of enantiomeric product alcohols, particularly when the samples are available in only very low concentrations in biological fluids. A prime objective of these investigations was the adaptation of current chromatographic methodologies for the determination of the optical purity of small amounts of metabolic products.

Since most in vitro studies of hepatic drug metabolism are performed utilizing incubations of subcellular preparations, it has been pointed out that levels and patterns of drug metabolizing activities with these systems are subject to a high level of external control. Because incubation conditions have been more or less standardized over past years, few attempts have been made to determine those conditions which provide for optimal enzyme activity. In view of this, it was informative to examine those biochemical factors which are critical in in vitro metabolism investigations.

Although the principal investigations of drug metabolism have centered around the use of hepatic tissue, it has become increasingly apparent that xenobiotic metabolism in non-hepatic tissues is of a greater significance than that assigned formerly. A preliminary investigation was undertaken to determine to what extent cerebral metabolism influences the disposition, in rat brain tissue, of several ip. administered sympathomimetic amines. A primary concern of this study was the availability of selective and sensitive analytical methodologies which would permit quantitation of trace levels of metabolites, including phenolic amines, in biological fluids.

The metabolism of xenobiotics in the multicellular organism has been studied by a variety of methods. Of great interest over the past several years have been attempts to develop tissue culture procedures utilizing isolated hepatocytes for these studies. The potential of hepatocyte cultures is somewhat different from systems involving broken cell preparations. Practically, however, because of the disadvantages, techniques involving cell cultures have not gained widespread acceptance. A principal requirement for cell cultures is the ability to prepare suspensions of viable, intact hepatocytes in high yields. However, because it has not been possible to sustain cultures of liver parenchymal cells in which drug metabolizing systems remain active through several generations, objectives lie in the greater maintenance of the differentiated cell state by suitable manipulation of the medium. An extensive study of conditions which would hopefully extend the xenobiotic metabolizing activity of hepatocytes in primary culture, and a preliminary assessment of their usefulness in conventional metabolism studies, was undertaken.

3. LITERATURE SURVEY

3.1 METABOLIC CARBONYL REDUCTION

3.1.1 Introduction

By far the greatest effort in the investigation of the drug metabolism process has been directed towards learning more about the biochemical nature of the microsomal mixed function oxidase system. This is not surprising in view of the fact that oxidative pathways dominate the biotransformation of drugs and other foreign compounds. This should not be interpreted, however, to mean that the remaining metabolic routes such as reduction, hydrolysis, phase II conjugations, and various other reactions are of any less importance. Even though these pathways have not been as extensively studied as microsomal oxidations, they can perform significant roles in the fate of xenobiotics in biological systems. For instance, carbonyl reductases have been found to exhibit substantial activity in rabbit and human tissue (9, 10), yet relatively little is known about this category of enzyme. While there does exist a creditable amount of knowledge about certain aldehyde reductases based almost solely on their physiological role, there is a comparable absence of specific information on the metabolic reduction of ketone-containing compounds. What follows is a summary of the available information on carbonyl reductases, with emphasis on ketone reduction. Also provided is an overview of the mechanisms involved during metabolic reduction.

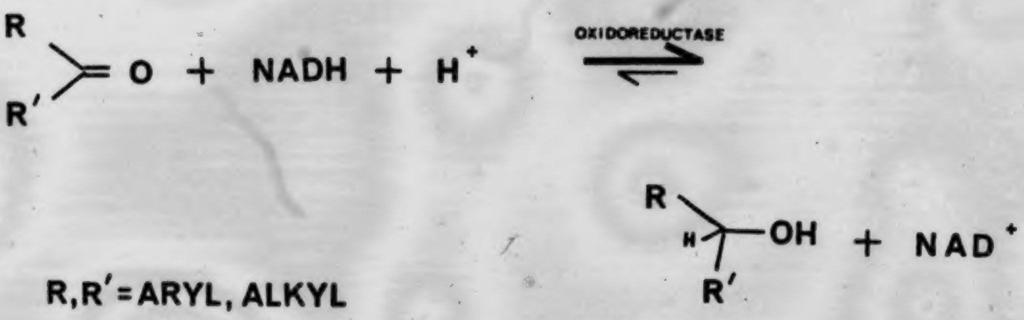
3.1.2 Aldehyde and Ketone Reductases

The metabolic reduction of aldehyde- or ketone-containing compounds is catalyzed by a rather diverse group of pyridine nucleotide-dependent enzymes located in the cytoplasm of mammalian cells (11, 12). But while associated with a specific biochemical function, this enzymic reaction is undoubtedly represented by a vast number of distinct reductases which differ within various sytems. For the most part, differentiation of carbonyl reductases has been historically based on their substrate specificities and the type of cofactor which served to mediate the reaction. Current research, however, is expanding the categorization of these enzymes with a greater emphasis being placed on their physical properties (13, 14).

Of the information available about carbonyl reductases, the major portion without doubt has pertained to the alcohol dehydrogenases (ADH) (15, 16), which are also referred to as either NAD-oxidoreductases or NAD-linked aldehyde reductases (NAD-AlR). While ADHs have diverse origins, mammalian tissue comprises the most extensive source of this enzyme (17). Of the large number of different alcohol dehydrogenases, the most comprehensively studied has been the enzyme located in horse liver (LADH), first isolated by Bonnichsen and Wassen (18) in 1948.

The term, alcohol dehydrogenases, originally referred to those NAD-dependent enzymes which exhibited a fairly broad specificity towards the oxidation of aliphatic primary alcohols. However, dehydrogenases, as do all carbonyl reductases, function as oxidoreductases (12, 19). Thus, these enzymes possess the distinctive capability of mediating both the reduction of carbonyl groups to the corresponding alcohols, as well as the reverse oxidation reaction (Fig. 1). The literature indicates however, that at equilibrium, reductase-catalyzed reactions definitely favor formation of the alcohol rather than the carbonyl compound (12).

FIGURE 1. Schematic representation of the metabolic interconversion between carbonyl containing compounds and their corresponding alcohols, as mediated by an oxidoreductase enzyme.



Any existing function for alcohol dehydrogenases in the drug metabolism process is, at present, unclear. Few compounds which could be classified as xenobiotics are known substrates for LADH. One notable exception to this generalization is the ability to catalyze reversibly the interconversion of cyclohexanone and cyclohexanol (24, 25). Alcohol dehydrogenases appear to be restricted to a more physiological role, reacting preferentially with endogenous substrates, such as short chain aliphatic aldehydes, ω-hydroxylated fatty acids, and various keto-steroids (20-23).

A group of enzymes similar to ADHs in that they mediate the reversible reduction of aldehydes have also been isolated from mammalian tissue (26-30). These reductases are distinct from the previously described NAD-linked dehydrogenases in that they can only utilize NADP as cofactor. Furthermore, the specificity of these aldehyde reductases (NADP-AlR) included not only various expected physiological compounds as substrates, but also a number of xenobiotic aromatic and aliphatic aldehydes (9, 10, 36, 38, 40). In many instances these enzymes could be isolated in highly purified forms for testing, which demonstrated that more than one distinct kind of NADP-AlR could be found in a single type of mammalian tissue (26, 31-35, 38, 40, 41). As was noted with the alcohol dehydrogenases, NADP-linked AlRs were also generally inactive toward ketones.

Ketone reductases, although related in function to the aromatic-aliphatic aldehyde reductases and the classical alcohol dehydrogenases, have been shown to exist as a quite distinct class of enzymes. Unfortunately, lack of specific information has precluded definitive characterization of these ketone reductases as a group. Very few enzymes responsible for this reaction have been isolated in spite of an increasing effort made towards investigating the reduction of xenobiotic ketones. Culp and McMahon (42) were able to partially purify an NADP-linked aromatic aldehyde-ketone reductase from rabbit kidney cortex. However, they did not examine any of its physical properties; based on its rather weak ketone specificity when compared to its capacity to reduce aldehydes, it was doubtful whether this enzyme was in fact any different from the typical aldehyde reductases described earlier. Several other aromatic aldehyde-ketone reductases have also been reportedly isolated from various species and characterized (28, 35, 36, 40), although no clear-cut classification of these enzymes could be made. Any proper classification was complicated because of the broad substrate specificities demonstrated by these reductases, which were found to overlap those of previously described NADP-linked mammalian AlRs (26, 31-34). For those aldehyde-ketone reductases which have been evaluated with xenobiotics, the activity towards ketones was relatively insignificant.

The single confirmed example of a specific ketone reductase was reported by Fraser et al. (43). They described an NADP-dependent enzyme isolated from dog erythrocytes and human liver which catalyzed the reduction of only α, β —unsaturated ketones. Although most, but not all of the α, β —unsaturated ketones tested were reduced, none of the saturated aldehydes or ketones examined was an active substrate, even though several of these compounds were shown to be reduced by some of the previously described reductases (ADH, NAD-AlR, NADP-AlR).

3.1.3 Role of NADPH and NADH in the Drug Metabolism Process

3.1.3.1 Microsomal Oxidation

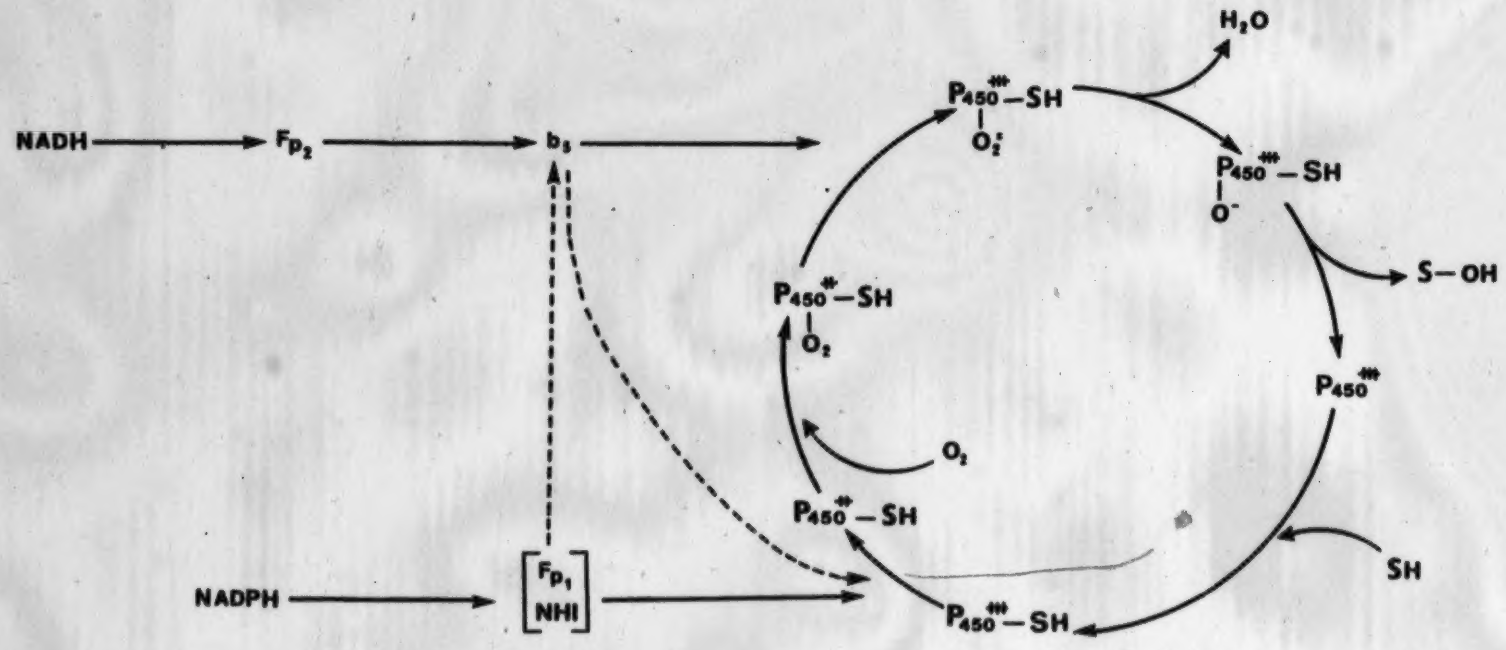
3.1.3.1.1 NADPH/NADH in Reconstituted Enzyme Systems

The function of NADPH as a donor of the reducing equivalents (electrons, hydrogen) required during the microsomal oxidization of steroids, drugs, and other xenobiotics, has been thoroughly documented (44-49). Mueller and Miller (50, 51) originally demonstrated that in vitro metabolic hydroxylation required NADP$^+$, molecular oxygen and both the microsomal and soluble fractions of liver homogenate. Later Brodie et al. (52) showed that oxidative activity actually resided in the microsomal fraction, and the requirement for the soluble fraction could be replaced by either adding NADPH directly or by addition of an NADPH-generating system consisting of NADP$^+$, glucose-6-phosphate, Mg^{++}, and glucose-6-phosphate dehydrogenase. By requiring both oxygen and a reducing agent for the metabolic reaction, the catalyzing enzyme was classified as a mixed function oxidase (MFO) or monooxygenase (53).

Besides establishing the function of NADPH, these initial investigations indicated a possible role for NADH as well (51, 54). While not confirmed until later (55-57), the investigators were essentially correct in proposing that NADH could similarly serve as an electron donor in the NADPH-linked MFO system (Fig. 2). But even though it was demonstrated that NADPH, which was originally considered absolutely necessary for microsomal oxidation, could be replaced with NADH, of the two cofactors NADPH was the significantly more efficient electron donor (44, 54). Substitution of NADPH with NADH as the sole electron source effectively reduced the rate of product formation by 80-90% (58-71).

Microsomal oxidation is a two electron process which operates through a pair of interacting electron transport chain systems which are linked with the cytochrome - P_{450} reduction-oxidation cycle (Fig. 2). The first, an NADPH-linked system (72-74), supplies one electron to reduce the oxidized cytochrome P_{450} substrate complex ($P_{450}{}^{+++}$-SH). This first electron is derived almost exclusively from NADPH (75, 76). The second transport mechanism is an NADH-linked system (77, 78) involved in the reduction of the oxygenated-reduced cytochrome P_{450}- substrate complex (O_2-$P_{450}{}^{++}$-SH), through which a second electron is transferred via cytochrome b_5. While this second transport system is able to receive a reducing equivalent from NADPH when it is the only available donor (79, 80), NADH appears to be the preferred donor of the

FIGURE 2. Schematic representation of the proposed interactions of the NADH- and NADPH-dependant electron transport chains involved in the cytochrome-P_{450} reduction - oxidation cycle associated with metabolic microsomal oxidation reactions. [Adapted from Hildebrandt and Estabrook, (57)].


NADH
F_{p_2}
b_5
$P_{450}^{+++}-SH$ / O_2^-
H_2O
$P_{450}^{+++}-SH$ / O^-
S—OH
P_{450}^{+++}
SH
$P_{450}^{+++}-SH$
$P_{450}^{++}-SH$
O_2
$P_{450}^{++}-SH$ / O_2
NADPH
F_{p_1}
NHI

Fp_1- NADPH-cytochrome c reductase; Fp_2- NADH-cytochrome b reductase;
NHI- Nonheme iron-containing protein; P_{450}- cytochrome P_{450};
cyt b_5- cytochrome b_5; SH- substrate; SOH-hydroxylated substrate.

second electron when both pyridine nucleotides are available (63-65, 68, 81, 82).

3.1.3.1.2 NADH-Synergism

Mueller and Miller (51) were first to recognize that NADPH-catalyzed microsomal oxidation reactions could be substantially enhanced by the concurrent addition of NADH. In some instances the increase exceeded the additive effects of the actions of the two cofactors. It has since been demonstrated that with the availability of both cofactors, reaction rates could be 30-100% higher compared to when NADPH was the only coenzyme (58, 59, 60-68, 71). Reasons initially proposed to explain this observation were rapidly dismissed (60, 64, 81). These included a) the conversion (transhydrogenation) of NADH to NADPH, and b) the suggestion that NADH prevented NADPH from being utilized in various competing reactions. It was eventually proposed by Cohen and Estabrook (60) that the synergistic effect was in fact due to a cooperative interaction which existed between the two microsomal electron transfer chains. This interaction, involving cytochrome-P_{450} and cytochrome b_5, made it possible to utilize NADH more efficiently during oxidative reactions (57). Correia and Mannering (63-65) supported this concept by proposing that NADH is a better source of the second electron than NADPH, and provided a larger pool of electrons at cytochrome b_5. Subsequent studies confirmed this theory (68, 81). The exact details concerning the transport of electrons and of NADH-synergism are still far from being completely understood.

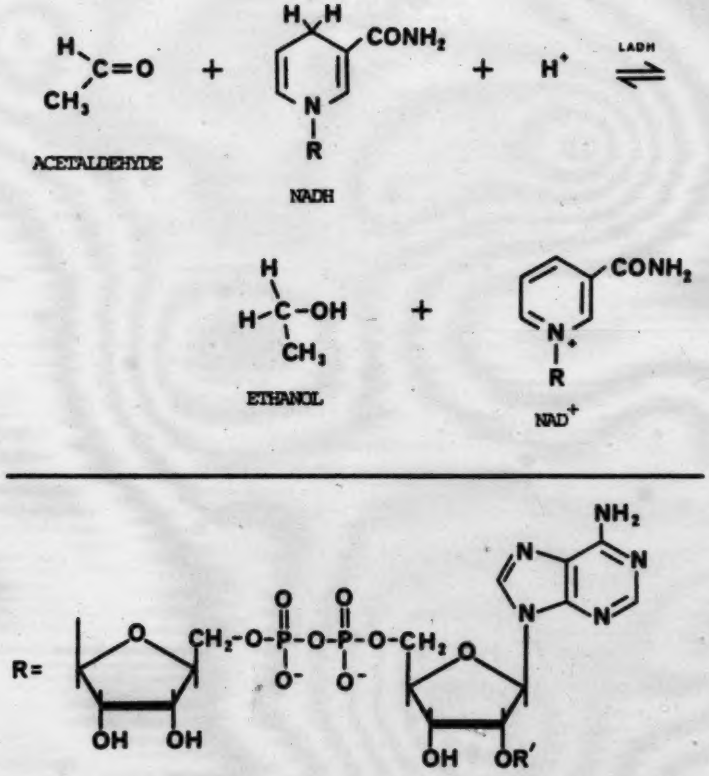
2.1.3.2 Cytoplasmic Carbonyl Reduction

2.1.3.2.1 Mechanism of Hydride Transfer

In general, very little direct information is available on the ketone reductases. It is possible however, for certain conclusions to be drawn about the mechanisms by which they function, based on their similarities with the more extensively studied liver alcohol dehydrogenases (LADH).

Early investigators of the ADH catalyzed oxidation of ethanol to acetaldehyde (Fig. 3) tended to look upon the mechanism of this reaction in the same manner in which they viewed mixed function oxidative reactions; that is, solely in terms of electron transfer (83). Any loss or gain of hydrogens which was demonstrated by the stoichiometry was thought to occur through an exchange with the surrounding medium. It was, therefore, significant when Vennesland and Westheimer (83) using isotopically labeled NADH (NAD$\underline{D}$), found that during enzymic reactions mediated by LADH complete transfer of deuterium from NAD$\underline{D}$ to the oxidized substrate occurred (or from the reduced substrate to the oxidized cofactor) (Fig. 4). The resulting alcohol contained one atom of

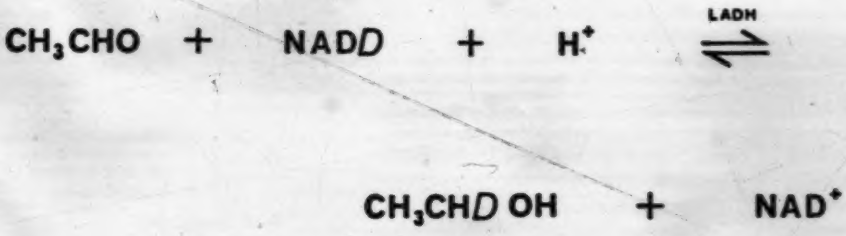
FIGURE 3. NAD-linked alcohol dehydrogenase mediated interconversion of acetaldehyde and ethanol.


ACETALDEHYDE
NADH
H⁺
LADH
ETHANOL
NAD⁺
R=

NAD $R' = H$
NADP $R' = PO_3^{=}$

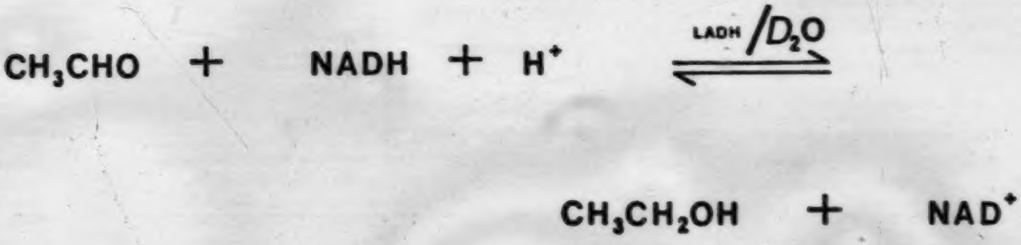
deuterium in the methylene group. The same reaction, but

FIGURE 4. Metabolic interconversion of acetaldehyde and ethanol in the presence of deuterium labeled NADH (NAD*D*). [Adapted from Vennesland and Westheimer (83)].


$CH_3CHO + NAD\mathit{D} + H^+ \xrightleftharpoons{LADH}$
$CH_3CH\mathit{D}OH + NAD^+$

with nondeuterated-NADH was carried out in D_2O (Fig. 5). The direct transfer of the hydrogen from NADH was confirmed

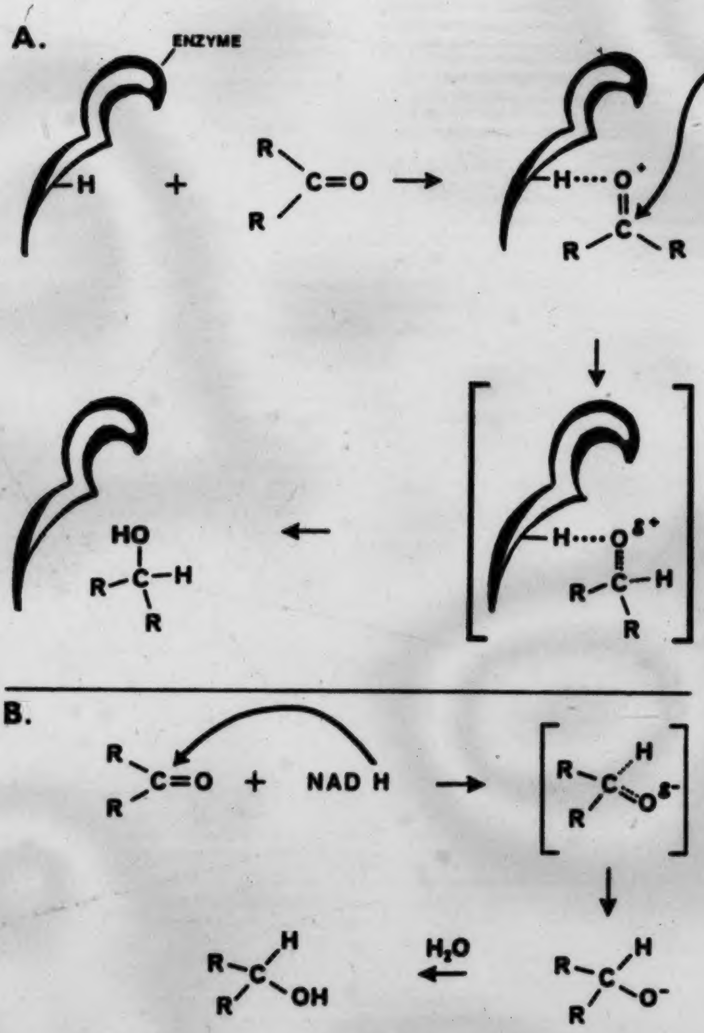
FIGURE 5. Metabolic reduction of acetaldehyde in the presence of deuterium labeled H_2O (D_2O). [Adapted from Vennesland and Westheimer (83)].


$CH_3CHO + NADH + H^+ \xrightleftharpoons{LADH/\mathit{D}_2O}$
$CH_3CH_2OH + NAD^+$

when the product alcohol was found to contain no deuterium. This ruled out any possibility that the hydrogen atoms introduced into the product were derived from the surrounding aqueous medium.

The reduction of a carbonyl compound to an alcohol requires the equivalent of two hydrogen atoms. The mechanism by which this reaction occurs involves the addition of a hydride ion (H^-) and a proton (H^+) (19). The reduced pyridine nucleotide (NADPH/NADH) serves as the source of the hydride ion (Fig. 3), but the origin of the proton in the equilibrium reaction has yet to be resolved. Schellenberg (84) suggested that this proton could arise from appropriate functional groups on the enzyme. Through specific interactions between protein and substrate, it was possible to activate the carbonyl by either direct protonation, or alternatively through hydrogen bonding with a protonated group on the enzyme (Fig. 6A). As a result of this prior protonation of the carbonyl oxygen, the protonated species ($R_2C{=}OH^+$) is more susceptible to a nucleophilic attack than the non-protonated ketone ($R_2C{=}O$). The nucleophile is the hydride ion (H^-) from NADH, which does not have to compete with protons (H^+) from the surrounding water. Based on this proposed mechanism, Schellenberg was able to explain why no exchange of protons with the medium occurred during enzymic reduction. In comparison, transfer of the hydride ion directly to the carbonyl carbon of $R_2C{=}O$ would result in the incorporation of a proton from H_2O (Fig. 6B).

FIGURE 6. Plausible mechanisms for the metabolic reduction of carbonyl-containing compounds: A) nucleophilic addition following protonation of the cabonyl oxygen through specific enzyme - substrate interactions; B) direct nucleophilic addition without prior protonation of the oxygen. [Adapted from Schellenberg (84)].

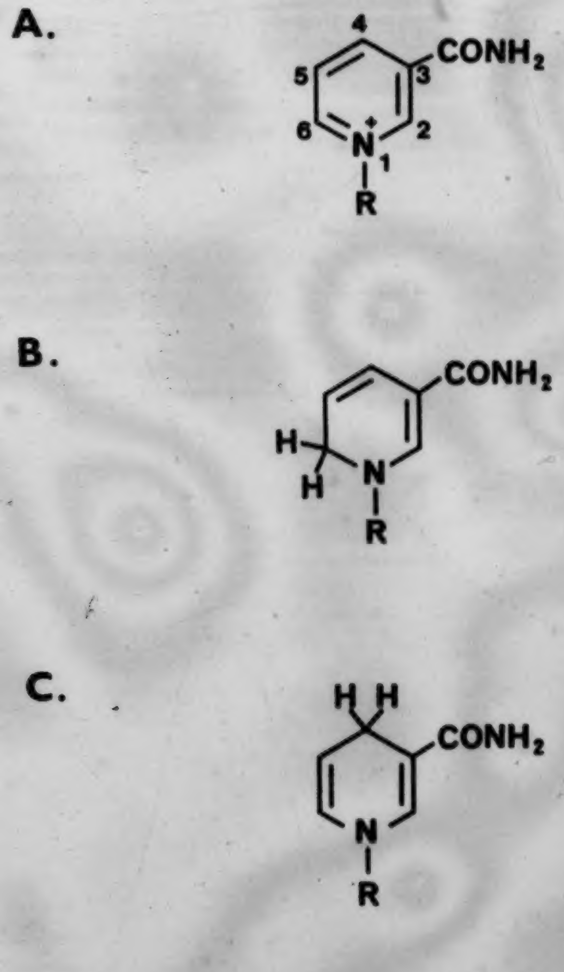

A.
ENZYME
H
R
C=O
R
H····O⁺
C
R
R
NAD H
H····O δ+
R–C–H
R
HO
R–C–H
R
B.
R
C=O
R
NAD H
R
C
H
O δ-
R
H
R–C
R
O⁻
H₂O
R–C
H
R
OH

3.1.3.2.2 Structural and Functional Relationship of NADPH/NADH

3.1.3.2.2.1 The Structure of Pyridine Cofactors as Pertains to Active Site

Considerable effort has been devoted to elucidating the structure-function relationship of the cofactors (NADPH/NADH) utilized during enzyme mediated carbonyl reductions. Karrer *et al*. (85), using *N*-methylnicotinamide analogs as model compounds for NADH (Fig. 7A), originally proposed that reduction of NAD^+ occurred at the pyridine moiety at one of the positions *ortho* to the nitrogen atom (Fig. 7B). This claim was further supported by Knox and Grossman (86) who suggested that the reduced cofactor was the 1,6-dihydro derivative. It was not until Pullman *et al*. (87-89) carried out a comprehensive series of enzymic and chemical reactions that the *ortho* and *meta* positions on the pyridine ring were eliminated as possible active sites. This provided the first indication that the transfer of hydrogen actually occurred at the *para* (*C*-4) position of the nicotinamide ring. Pullman and coworkers concluded that the reduced cofactor, NADH, was probably a 1,4-dihydropyridine compound (Fig. 7C). This proposal was corroborated by Loewus *et al*. (90, 91) who found that when the NADH used in an ADH catalyzed reduction was substituted with either 2-, 3-, or 4-monodeuterated NADH, only in the presence of the 4-deuterio-NADH did the resulting pyridine product contain deuterium. Unequivocal evidence that the C-4 position of the pyridine ring was indeed the site of active hydrogen transfer was submitted by Dubb et al (92) and Hutton and Westheimer (93) who employed NMR techniques to confirm Pullman's earlier conclusion.

FIGURE 7. Proposed sites of hydrogen transfer occurring at the pyridine moiety of associated coenzymes during oxidoreductase mediated reactions: A) oxidized form of coenzymes and analogs; N-methylnicotinamide, R = CH$_3$; NADP, R = see Fig. 3; NADP, R = see Fig. 3. B) *ortho*- or 1,6-dihydro reduced form. C) *para*- or 1,4-dihydro reduced form.



3.1.3.2.2.2 Stereochemistry of NADPH/NADH

Because of the presence of the carboxamide group, the two hydrogens *para* to the pyridyl nitrogen of the reduced nicotinamide moiety are not enzymatically equivalent. Thus, when NADP/NAD binds to an enzyme, only one side is able to donate (or receive) the transferred hydrogen. Recognized as enantiotopic, these hydrogens were arbitrarily designated as H_A (α-hydrogen) and H_B (β-hydrogen) (Fig. 8), although the meaning of the A and B faces of the nicotinamide moiety in terms of absolute configuration was not deduced until years later. Cornforth *et al.* (94, 95) devised a method for the chemical degradation of NADH in which positions 4 and 5 could be isolated as succinic acids without affecting the stereochemistry of the two hydrogens at position 4 (Fig. 9). Subsequently, this sequence of reactions was applied to *C*-4 monodeuterated cofactors and the resulting monodeuterio

FIGURE 8. Stereochemical representation of the reduced nicotinamide moiety of NADPH/NADH. (R = see Fig. 3).

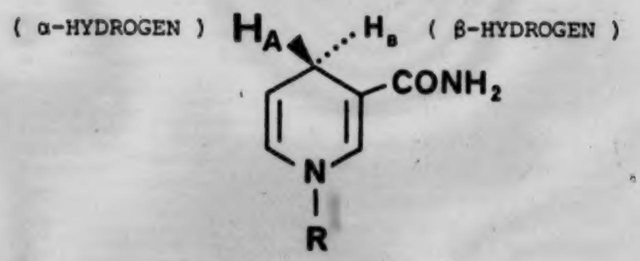
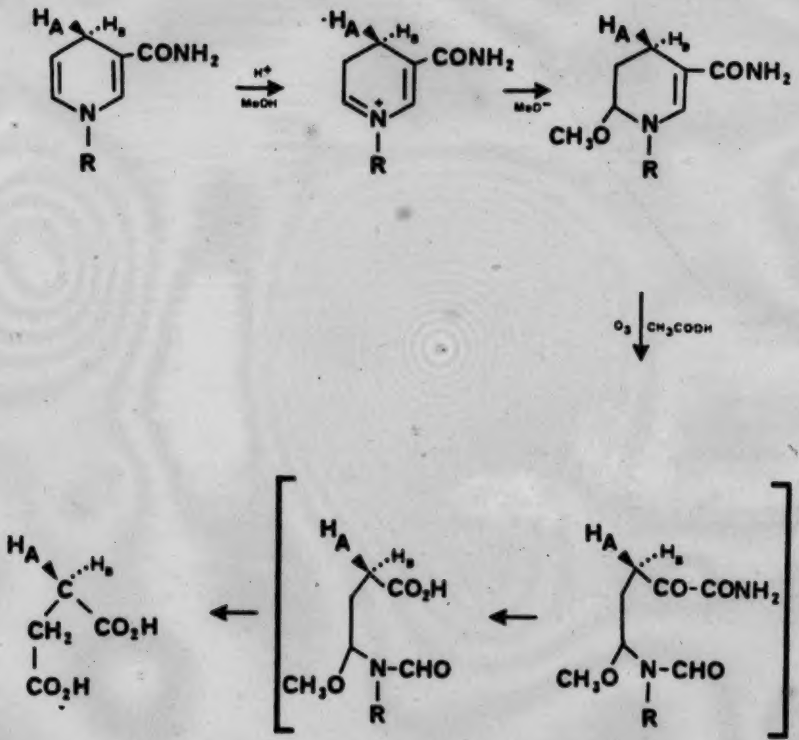


FIGURE 9. Degradation of the 1,4-dihydronicotinamide moiety to succinic acid with retention of the stereochemistry of the two hydrogens at the position *para* to the pyridyl nitrogen. The method is equally applicable to 1,4-dihydro-1-methylnicotinamide (R = CH_3) and to reduced nicotinamide adenine dinucleotide coenzymes (NADPH, NADH: R = see Fig, 3). [Adapted from Cornforth *et al* (94, 95).



H_A
H_B
CONH₂
N
R
H⁺
MeOH
MeO⁻
CH₃O
O₃
CH₃COOH
CO-CONH₂
N-CHO
CO₂H
C
CH₂

succinic acids derived from the A-deuterio and B-deuterio NADH compounds were found to correspond to the R and S configurations, respectively. As a deuterium at the α position resulted in the R-isomer, the hydrogen H_A was labeled as the pro-R hydrogen. By the same reasoning H_B was tagged the pro-S hydrogen. Nakamoto and Vennesland (96) have since shown that the pro-R and pro-S sides of NADH correlate to the stereochemistry of that found with NADPH. Therefore, all rules which pertain to NADH, generally apply to NADPH as well.

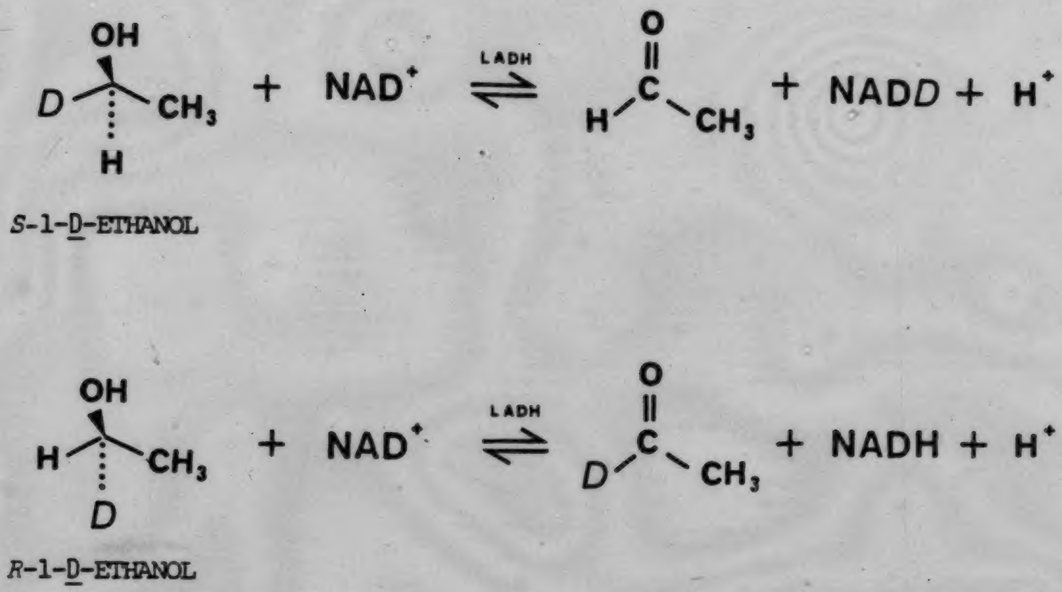
The hydrogen stereospecificities of carbonyl reductases have been studied for a number of aldehyde reductases and closely related enzymes. Even prior to confirmation of the absolute configuration of NADPH/NADH, it was proposed that these enzymes acted in a stereochemical manner by activating preferentially either one of the hydrogens on the reduced cofactor. Vennesland and Westheimer (86) reported that ADH utilized the H_A side of NADH when converting acetaldehyde to ethanol. Examinations of other dehydrogenases demonstrated a similar stereospecificity, regardless of whether NADH, NADPH or both co-enzymes were utilized (97, 98). Those dehydrogenases which had the same cofactor specificity as did ADH became known as A-side specific, whereas those with the opposite stereospecificity were termed B-side specific enzymes.

In contrast to the A-specificity of alcohol dehydrogenases (99) and aldehyde reductases (29, 41), the majority of ketone reductases demonstrated a B-side (pro-S) stereospecificity. Culp and McMahon (42) studied the reduction of p-chlorobenzaldehyde with a partially purified aromatic aldehyde-ketone reductase, isolated from rabbit kidney. By making use of tritiated NADH (NADT), it was clearly shown that this isolated reductase utilized the β-hydrogen. Felsted *et al.* (100) examined the cofactor specificity of several rabbit liver carbonyl reductases and found that the enzymes which reduced daunorubicin, metyrapone, and oxisuran, all displayed B-side specificity. One exception was dihydromorphine reductase, which was α-hydrogen stereospecific.

3.1.3.2.3 Stereochemistry of the Products of Reduction

The transfer of hydrogen which accompanies the reversible reduction of carbonyl compounds has been shown to be stereospecific not only with respect to cofactor, but with regard to substrate as well. Using R- and S-1-D-ethanol, Loewus *et al.* (90) demonstrated only one specific hydrogen atom was always transferred from substrate to coenzyme by ADH (Fig. 10). In just the opposite manner, Levy *et al.* (101)

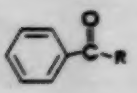
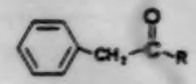
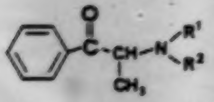
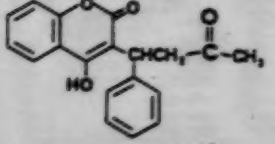
FIGURE 10. Scheme of the stereospecific transfer of hydrogen (deuterium, *D*) between cofactor and substrate during the metabolic oxidation of alcohols (or reduction of carbonyl containing compounds). [Adapted from Loewus *et al* (90, 91) and Levy *et al* (101)].



and Loewus (91) observed that 1-D-ethanol produced by enzymic reduction could only be isolated as the S-isomer. Alcohol dehydrogenases are not unique in exhibiting stereospecificity towards the substrate. There are several examples of ketone-containing compounds undergoing stereoselective reduction, both *in vivo* and *in vitro*. Smith *et al.* (102) reported that a number of arylalkylketones orally administered to rabbits were asymmetrically reduced to the corresponding alcohols. Recovered as the glucuronide conjugates, all the alcohols were shown to be exclusively the *S*-isomers (Table 1). Several medicinally important compounds possessing a ketone group have also been reported to be reduced stereoselectively *in vivo*. Lewis *et al*. (103) examined the metabolic fate of warfarin, an oral anticoagulant, in man. Both the *R*- and *S*-isomers were reduced almost entirely to the corresponding *S*-alcohols (*RS*- and *SS*-diastereoisomers of warfarin alcohol respectively) (Table 1). Other ketone-containing drugs observed to be asymmetrically reduced include oxisuran (104, 105), methadone (106), diethylpropion (107), and naltrexone (108).

Beckett, Testa, and Mihailova (109-111) examined in detail the carbonyl reduction of the anorexic agent, diethylpropion, and related amino-ketones in man. They demonstrated that the stereochemistry of the products was influenced by the configuration of the groups adjacent to the carbonyl moiety, as well as the relative size of these groups. A summary description of the extent to which product stereoselectivity occurred with the substrates tested (*N*,*N*-diethylpropion, *N*-ethylpropion, propion) is provided in Table 1. The effect on reduction caused by the nature of the groups attached to the carbonyl moiety was investigated in detail earlier by Prelog and coworkers (112-117). These studies resulted in a method by which the stereochemistry of carbinols produced by metabolic reduction could be predicted. By determining the absolute configuration of a large number

TABLE 1. Stereoselectivity of the Metabolic Reduction of Various Ketone Containing Compounds.

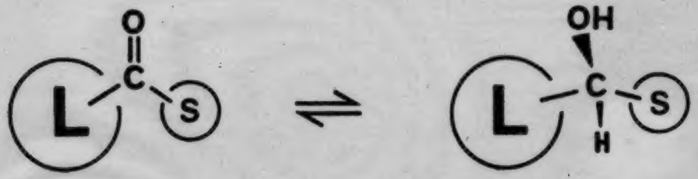
COMPOUND						
STRUCTURE	NAME	SPECIES[1]	ENZYME[2]	PREDICTED CONFIGURATION OF PRODUCT ALCOHOL	ACTUAL CONFIGURATION OF PRODUCT ALCOHOL	(REF.)
 R CH_3	ACETOPHENONE	Rb	AROMATIC KETONE CORTEX REDUCTASE	*S*	76% *S*/24% *R*	442
		Rb	*IN VIVO*	*S*	100% *S*	102
CH_2CH_3	PROPIOPHENONE	Rb	*IN VIVO*	*S*	100% *S*	102
$CH_2CH_2CH_3$	BUTOPHENONE	Rb	*IN VIVO*	*S*	100% *S*	102
 R CH_3	PHENYLACETONE	Rb	*IN VIVO*	*S*	100% *S*	102
CH_2CH_3	1-PHENYL-2-BUTANONE	Rb	*IN VIVO*	*S*	100% *S*	102
 R^1 R^2						
CH_2CH_3 CH_2CH_3	N,N-DIETHYLPROPION	Mn	*IN VIVO*	*S*	80% *S*/20% *R*	109
CH_2CH_3 H	N-ETHYLPROPION	Mn	*IN VIVO*	*S*	66% *S*/34% *R*	109
H H	PROPION	Mn	*IN VIVO*	*R*	15% *S*/85% *R*	109
						
S - ISOMER	WARFARIN	R	LIVER CYTOSOL REDUCTASE	*S*	77% *S*/23% *R*	510
		Mn	*IN VIVO*	*S*	57% *S*/43% *R*	510
R - ISOMER	WARFARIN	R	LIVER CYTOSOL REDUCTASE	*S*	85% *S*/15% *R*	103
		Mn	*IN VIVO*	*S*	90% *S*/10% *R*	103

[1] Rb = RABBIT, Mn = MAN, R = RAT

[2] *S* = SINISTER, *R* = RECTUS

of alcohols obtained by microbially-mediated reductions, Baumann and Prelog (113) observed that the product stereospecificity of the reaction could be represented by the simple scheme illustrated in Figure 11. In this figure, L

FIGURE 11. Prelog's Rule for Preferred Stereospecific Reduction, (L = large group; s = small group). [Adapted from Baumann and Prelog (113)].



(large) and s (small) represent respectively, a bulky and a small group adjacent to the carbonyl moiety. It was postulated that if the ketone is positioned so that the larger substituent is placed on the left, reduction predominately occurs such that the resultant hydroxyl group rises above the plane of the molecule. This principle was reinforced further by others (111, 118, 119), although estimations of the relative sizes of the groups bordering the ketone are for the most part made solely on the basis of empirical observations.

By applying the above formula, the enantioselectivity of the mammalian reduction of various ketones could be rationalized (Table 1). Observed configurations of the resultant alcohols generally conformed to that predicted by Prelog's Rule. Although the in vitro reduction of S-warfarin by rat liver cytosol occurred with a lesser degree of stereoselectivity than would otherwise be expected, the configuration of the alcohol isomer produced in slight excess, was qualitatively correct.

3.1.4 The Function of Carbonyl Reductases

3.1.4.1 Physiological Role of Reductases

Although carbonyl reductases are abundant in mammalian tissue, their exact in vivo functions are mainly unclear. With alcohol dehydrogenases (ADH) and aldehyde reductases (AlR) their role is perhaps predominately in the maintenance of normal cellular physiology (13, 36, 100, 120-123). Most reductases have been shown to demonstrate some reduction potential beyond that of an endogenous role (i.e. the capacity to reduce simple compounds not associated with a biochemical function), but very few drugs have actually been found which are actively reduced by these reductases. This suggests that ADHs and AlRs possess functions too specific in nature to serve as drug-metabolizing enzymes. In contrast, ketone reductases are generally regarded as non-specific in their action. This is based on the extensive ketone reductase activity found in most mammals (10, 12) and the capability of ketone reductases to react readily with aldehyde substrates as well as ketone compounds (13, 84, 100). Identification of a more conclusive role for the ketone reductases other than xenobiotic metabolism awaits further studies with purified enzymes.

3.1.4.2 Pharmacological Influence of Reductases Upon Administered Drugs

In contrast to a drug undergoing metabolic oxidation with the elimination of pharmacological activity being the most frequent consequence, the conversion of ketone- containing drugs to alcohol metabolites usually results in the retention of pharmacological activity. In many instances it is the alcohol-containing metabolite which actually provides the desired response. Sullivan et al. (106) found, for example, that most of the analgesic activity demonstrated by methadone could be attributed to the enzymatically produced alcohol metabolites α-l-methadol and α-l-N— demethylmethadol. Di Carlo et al. (104) suggested that the immunosuppressive effect of oxisuran was due, in part, to its reduction to oxisuran alcohol. Animals that readily metabolized oxisuran to the alcohol demonstrated significantly prolonged survival times of skin grafts when compared to animals that produced less oxisuranol. Several other examples of pharmacologically active alcohol metabolites have also been reported. Roering *et al.* (124) showed that 6-β-naltrexol and 6-β-naloxol contributed to the action of the parent compounds, naltrexone and naloxone, respectively, and Hoffman and Bachur (125) noted that daunorubicin alcohol not only possessed the same cytotoxic activity as its parent, but had a longer plasma and urinary half-life as well. An increase in half-life with retention of activity was similarly observed with the reduced metabolite of metyrapone (126). Only with the reduction of warfarin was a loss in pharmacological action noted (103). Metabolism of either *R*- or *S*-warfarin to the corresponding alcohols resulted in products which had only weak anticoagulant activity.

3.2 EXTRAHEPATIC DRUG BIOTRANSFORMATION AND THE ANALYSIS OF TRACE METABOLITES

3.2.1 Introduction

Research into the complex nature of hepatic drug metabolizing enzymes has been making steady advancement, but while the liver is the primary site for biotransformation of most xenobiotics, it is not the only site. Depending on the properties of the drug, its route of administration, and its distribution in tissue, metabolism may occur in non-hepatic tissue as well. Indeed, many extrahepatic organs including kidney, lung, skin, placenta, gastro-intestinal tract, brain and blood, have been found to contain active drug metabolizing systems. In many instances, the enzyme system closely resembles that observed in the hepatic system, but in other cases, significant differences do exist. Furthermore, numerous studies have reported that certain enzyme activities in various non-hepatic tissues are equal to, or greater than that found in hepatic tissue (127-131) although the comparably larger size of the liver makes it overall a much more efficient drug metabolizing system. Yet despite the many interesting aspects of metabolism in tissue other than hepatic, the pharmacological significance of these processes has only been marginally examined. Only a few general reviews on extrahepatic metabolism have been published (132-134), as well as several recent papers on pulmonary (127, 135) and kidney (127) metabolism, and biotransformation in the skin (136).

Probably the major obstacle to metabolism studies of extrahepatic tissue has been the lack of adequate experimental procedures which permit a distinction between metabolism occurring in vivo in specific non-hepatic tissues, and that arising from biotransformations by liver enzymes. For this reason the vast majority of investigations have utilized broken cell tissue preparations, making it difficult to properly interpret the role of non-hepatic enzyme systems. Still, by means of isolated organ perfusion studies (137-139) when possible, and by the adaption of tissue culture methodology to cells other than hepatocytes (3, 5, 140-142), the contribution of various enzyme systems to the overall fate of xenobiotics in the body is slowly being evaluated.

3.2.2 Role of Extrahepatic Drug Metabolism

Although non-hepatic metabolism of xenobiotics may not be significantly notable in all phases of drug disposition, it can substantially alter the biological fate and effects of drugs. But while these systems do not usually contribute markedly to the elimination of drugs by converting them to more water-soluble compounds, the observation that diverse tissue types possess the capability to metabolize xenobiotics suggests a possible physiological role for the activity.

Among the more active non-hepatic tissues are those associated with the introduction or elimination of xenobiotics, these tissues include skin, lung, gastrointestinal tract and kidney. Because of this, the most commonly accepted explanation for the process of extrahepatic metabolism is that it evolved as part of the body defense mechanism against natural xenobiotics and as such, is more extensive at sites of entry into the body (134). Furthermore, as such contact with environmental foreign compounds is generally under chronic conditions in which only trace amounts are involved, this accounts for the relatively low metabolizing capacity of these tissues. The role of extrahepatic biotransformation becomes of increased importance by the suggestion that 90% of all cancers may be of environmental origin (143, 144), and that highly reactive and potentially toxic intermediates are the result of the conversion of these compounds to more polar oxidative metabolites (145). This explanation does not however, account for metabolism occurring in such tissues as adrenals, testes or brain. In these instances, the process has possibly a physiological role, but of sufficiently low substrate specificity to allow for reaction with various related exogenous compounds (134). It is worth considering however, that the actual function of extrahepatic metabolism is situated between these two possibilities.

3.2.2.1 Metabolism of Drugs by Brain Tissue

Recently there has been a significant interest in examining the accumulation of various drugs in cerrebral tissue. This interest has arisen in particular, since, in many instances certain drugs and/or their metabolites have been found to exert some pharmacological effects within the brain region. Therefore, if brain tissue does metabolize xenobiotics, this capability might be best described as a localized process for the activation, alteration, or termination of pharmacological activity. Although the brain exhibits only a very weak degree of metabolism compared to the hepatic system, various examples of drug biotransformations have been found (Table 2) (132).

3.2.2.1.1 Amphetamine: The Relationship Between Metabolism and Response

There is considerable information available regarding the ability of amphetamines to alter the behavioural patterns of users. These changes and other pharmacological actions are believed to result from their actions on the release and re-uptake of catecholamines in nerve terminals in various tissues, particularily in brain (146-149). Fisher et al. (150) was the first to suggest that the metabolites

TABLE 2. Metabolic Reactions Detected in Brain Tissue. [Adapted from Gorrod (132)].

METABOLIC PATHWAY	SUBSTRATE
IN VITRO	
DEACETYLATION	N-Hydroxy-2-fluorenylacetamide
DESULFURATION	Thiopental
NITRO REDUCTION	*p*-Nitrobenzoic acid
DEHYDROXYLATION	N-Hydroxy-2-fluorenylacetamide
RING HYDROXYLATION	Amphetamine, N-*n*-Butylaniline
N-OXIDATION	Chlorpromazine
S-OXIDATION	Chlorpromazine
ISOLATED PERFUSED BRAIN *IN SITU*	
ACETYLATION	4-Aminoantipyrine
GLUCURONIDATION	Oxazepam
DEMETHYLATION	Aminopyrine

et al. (150) was first to suggest that the metabolites p- hydroxyamphetamine and p-hydroxynorephedrine may have an important role in causing the depletion of norepinephrine. Numerous investigators have since attempted to determine whether, and to what extent, the metabolites of amphetamine contribute to the response produced by the parent compound (147, 148, 151-153). In at least one species (rat) considerable effort has been made in quantitating the accumulation of these metabolites in various brain regions and other tissues following administration of amphetamine (148, 154-163). As a result, the role of both p-hydroxyamphetamine and p-hydroxynorephedrine as false transmitters have been implicated by collaboration of in vivo (147, 148, 155, 164-170) and in vitro (147. 154, 171-174) investigations. As with the uptake of dopamine (155, 172) both metabolites are stored in noradrenergic nerve terminals where they displace norepinephrine. As a result it appears that the tissue distribution of these metabolites determines the intensity of the response to an acute dose of amphetamine. Furthermore, variations in the metabolism pattern of amphetamine have been shown to occur with chronic usage, resulting in changes in the distribution of its active metabolites. These changes in the disposition of p-hydroxyamphetamine and p-hydroxynorephedrine have been implicated in the observed fluctuation in responsiveness to amphetamine which develops during chronic administration (155, 164-166, 169).

3.2.3 Analysis of Trace Metabolites in Biological Fluids

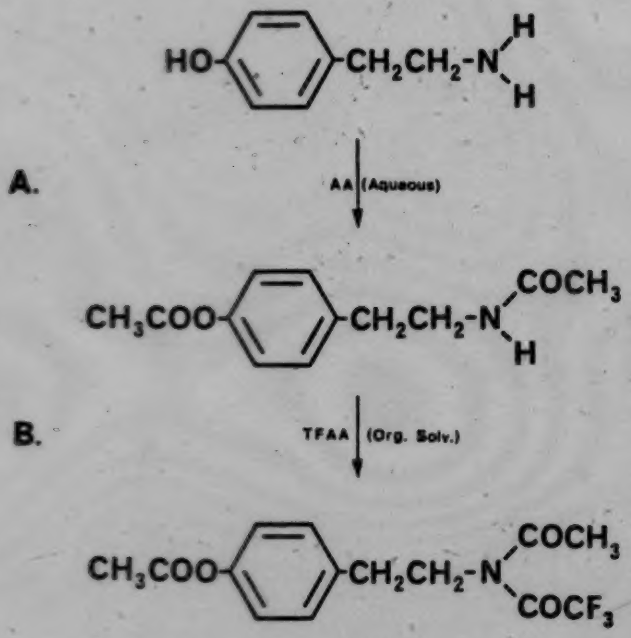
An important technique utilized in the investigation of drug action mechanisms has been the measurement of changes in the chemical composition of target tissues. Thus, the analysis of brain constituents after drug administration has become an experimental practice extensively used in neuropharmacology.

Various analytical procedures have been published for the determination of biogenic amines (catecholamines and tryptamine or phenylethylamine related compounds) in biological samples, with each of these procedures having advantages and disadvantages depending on their use. Although most analytical procedures were developed primarily for the measurement of catecholamine concentrations in brain tissue, the following survey of assay methods is confined to those procedures which have been adapted to the analysis of p-hydroxyamphetamine and p-hydroxynorephedrine. For example, early investigators obtained their results by spectrophotometric detection after the conversion of phenolic metabolites to fluorescent indole derivatives (175-177); another example being the oxidation of p-hydroxynorephedrine to p-hydroxybenzaldehyde (151, 176). However, because of the extremely small quantities of metabolites present in tissue, detection at trace levels was of primary importance. For this reason, most investigations have since relied on the use of tritium labeled amphetamine (148, 149, 151, 152, 154, 157, 161, 163, 178, 179). But besides being technically more complex and relatively non-specific, the major drawback with these radiolabeled assays is the high possibility of tritium exchange with tissue water. Groppetti and Costa (180) found that only 10% of the resulting radioactivity was identified with amphetamine and its metabolites. The remaining tritium was associated with the biological water and various brain components. This would lead to inaccurate results if quantitation was based solely on radioactivity measurements. Recently, the use of integrated ion current mass spectrometry has offered an alternative method which is both specific and extremely sensitive. Utilizing this technique, Danielson and coworkers (159, 160) were able to quantitate sub-nanogram amounts of p-hydroxyamphetamine in brain tissue following its recovery by ion exchange chromatography and formation of the dansylated derivative. Cattabeni et al. (158) described a similar use of mass fragmentography for the analysis of the hydroxylated metabolites of amphetamine. The residue obtained after drying a small aliquot (50-100 ul) of aqueous sample under a stream of nitrogen was reacted with pentafluoropropionic anhydride (PFPA) and the resulting derivatives screened by selected ion monitoring (SIM). In this way Cattabeni et al. reported the measurement of pmol quantities of p-hydroxynorephedrine in tissue samples.

Belvedere et al. (181) and Simpson (182) also employed perfluoroacylated derivatives of the phenolic metabolites for chromatographic analysis, but utilized gas liquid chromatography (GLC) with electron - capture (EC) detection as an alternative to the more expensive mass spectrometric methods. Derivatives were prepared by extracting the phenolic amines into an organic solvent following saturation of the aqueous sample with NaCl, and evaporating the organic phase to dryness. The ensuing organic residue was then reacted with an excess of perfluoroacylating reagent. Both investigators reported that levels down to 50 pg of the resultant derivatives could be detected when injected directly on-column (181, 182).

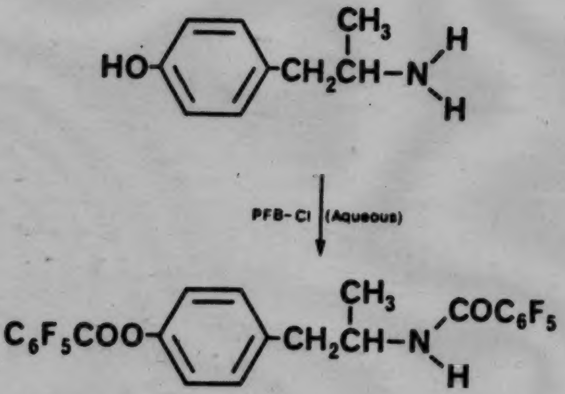
In these latter assay procedures mentioned above, utilizing mass spectrometry (158-160) and EC-GLC (181, 182), the sensitivity of the analyses compensated for the inefficient recoveries of the amphoteric metabolites from biological fluids. Recently, however, several investigators have relied on the process of acylating these biogenic amines, particularly phenylalkylamines, in situ in order to facilitate their extraction and analysis (183-185). This acylating procedure, achieved with acetic or propionic anhydride, can proceed in an aqueous medium and results in the formation of stable, lipophilic derivatives (Fig. 12A) which are extracted into organic solvents more efficiently than the parent compounds.

FIGURE 12. Scheme for the derivatization of *p*-tyramine: A) aqueous acetylation; B) trifluoroacetylation in organic solvent. (AA = acetic anhydride, TFAA = trifuoroacetic anhydride).


HO
CH_2CH_2-N
H
H
A.
AA (Aqueous)
CH_3COO
CH_2CH_2-N
$COCH_3$
H
B.
TFAA (Org. Solv.)
CH_3COO
CH_2CH_2-N
$COCH_3$
$COCF_3$

Furthermore, the acylated derivatives of most biogenic amines possess good chromatographic properties which permit their analysis by GLC. Martin and Baker (183) further reacted the acetylated compounds with perfluoroacylating reagents, producing stable derivatives which also possessed excellent EC detection sensitivities (Fig. 12B). Using this method they were able to routinely measure ng concentrations of biogenic amines in tissue samples. Cristofoli et al. (186) have since been able to perform a one-step aqueous acylation of phenolic primary amines with pentafluorobenzoyl chloride (PFB-Cl) and achieve similar results (Fig. 13). This was achieved by shaking an aqueous sample of p-hydroxyamphetamine with a mixture of pentafluorobenzoyl chloride in ethyl acetate/ acetonitrile, 9/1.

FIGURE 13. Derivatization scheme for the aqueous pentafluorobenzoylation of *p*-hydroxyamphetamine. (PFB-Cl = pentafuorobenzoyl chloride).


HO
CH3
CH2CH-N
H
H
PFB-Cl
(Aqueous)
C6F5COO
CH3
CH2CH-N
COC6F5
H

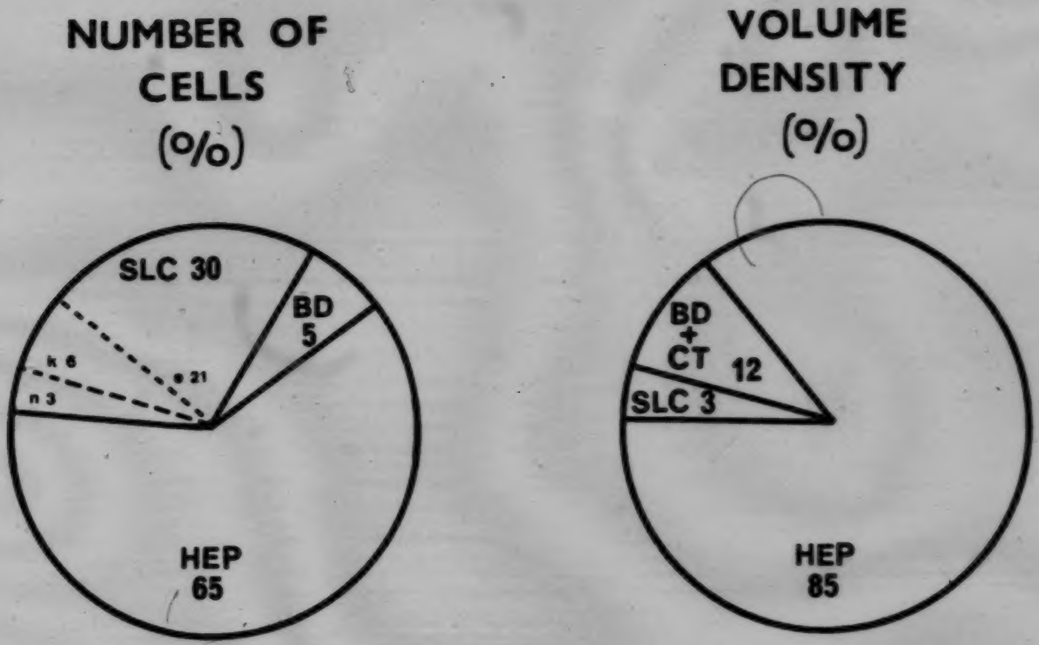
3.3 ISOLATED PARENCHYMAL HEPATOCYTES AS AN IN VITRO DRUG METABOLISM MODEL

3.3.1 Introduction

The increasing use of isolated mammalian cells, and in particular hepatocytes from mature animals, has contributed substantially to the understanding of cell physiology. Previous mention has been made of the advantages of, and also the problems associated with, the use of isolated liver cells (see Section 1.1.2.2.).

Although hepatocytes predominate in both volume and number, the liver contains various additional types of cells within its intrinsic structure (Fig. 14) (187). The focus of most studies, however, has been towards the separation of viable parenchymal hepatocytes and their maintenance in short term culture. Still, the utilization of isolated liver cells *in vitro* has been regarded as a particularly difficult problem for two major reasons: (i) the lack of efficient methods for the preparation of intact, viable cells, and (ii) the adult liver is normally a non-proliferating tissue. The first problem is a technical one which has essentially been solved. The second problem is a biological one and the major obstacle in isolated hepatocyte studies. Because of the inability of normal mature hepatocytes to proliferate, growth *in vitro* would not be expected. Even with their prolonged cellular life-span (188, 189), the maintenance of normal

FIGURE 14. The number and volume density of the different cell types found in adult rat liver tissue. [Adapted from Drochmans *et al* (187).



HEP : HEPATOCYTES
BD : BILE DUCTS
CT : CONNECTIVE TISSUE
SLC : SINUS LINING CELLS
  e - endothelial cells
  k - kupffer cells
  n - other non-parenchymal cells

functional hepatocytes in culture for more than a few weeks has proven unsuccessful (5, 190-218). As is typical with most highly differentiated epithelial cells, liver parenchymal cells either die or dedifferentiate early in culture (192, 209).

3.3.2 Preparation of Isolated Hepatocytes

3.3.2.1 Adult Rat Hepatocytes

Due to the lack of a satisfactory method for the preparation of viable hepatocytes, early tissue culture studies were limited to the explantation of aseptically prepared liver slices into an artificial in vitro environment (210). Although less than completely successful, this was an important first step. Subsequently, suspensions of isolated cells were obtained through the physical disruption of the tissue by various methods, including forcing the liver through cheese cloth (211), shaking with glass beads (212, 213), mincing with a tissue press (214), or by slow homogenization (215). Purification of the cells was accomplished by centrifugation. The recovery of intact cells, however, was usually less than 1% of the total liver, and the cells were inevitably damaged and of questionable viability. Although this was sufficient in instances where rapidly propagating cells were being used or cell lines being established (216, 217), these methods were severely restrictive for hepatic cell studies.

A crucial advancement in cell isolation occurred when it was recognized that intercellular Ca^{++} played an important role in the maintenance of the integrity of intact tissue (216-220). Similar claims were also made for Mg^{++} (216, 219) and K^{+} (221). It was reported that removal of Ca^{++} and Mg^{++} with chelating agents such as citrate (222-226) or ethylenediaminetetraacetic acid (EDTA) (210, 222, 225, 227-230) or chelation of cellular K^{+} with tetraphenylboron (TPB) (201, 221, 228, 231-235) prior to mechanical dispersion, facilitated the subsequent separation of cells. This resulted in higher yields of cells in apparently better morphological condition than was possible with mechanical disruption alone. Since these earlier studies, only Ca^{++} has remained a recognized variable because of its importance to a Ca^{++}-dependent adhesion factor found in all tissue (219, 236, 237). Further investigations have indicated that Mg^{++} (218, 219) and K^{+} (201, 222, 231, 237) do not really contribute to cellular cohesion and that the use of TPB is ineffective in promoting liver dispersion (231, 237, 238). In fact, it has recently been shown that the presence of K^{+} actually improved disruption of tissue (237) and that TPB is highly toxic towards mammalian cells (201, 221, 228, 231, 239).

The removal of Ca^{++} remains the only modification in the cationic environment which favors cell dispersion. Still, there is some reason to believe that liver cells prepared by methods involving chelation, regardless of whether mechanical treatment was also used, are structurally and functionally defective. Despite evidence that the isolated cells were technically intact, various disfunctions which included the impairment of protein (238), cholesterol (240), and RNA (241) synthesis, indicated numerous changes in cellular ultra-structure had in fact occurred. Furthermore, loss of vital membrane integrity was evident by the severe enzyme leakage displayed by these cells (189, 231, 241-247).

The introduction of collagenase as a liver dispersing enzyme greatly facilitated the preparation of intact viable cells. Shortly after Rodbell (248) demonstrated that adipose cells could be isolated by incubating fat tissue with collagenase, Howard and coworkers (249, 250) developed a procedure for preparing suspensions of isolated hepatocytes by incubating liver slices in the presence of a mixture of collagenase and hyaluronidase. This method was subsequently improved by Berry and Friend (251) and Howard et al. (252), who introduced a new technique involving in situ perfusion of the rat liver at room temperature with a similar collagenase/hyaluronidase buffer. By employing a continuous recirculating perfusion step prior to mincing and incubating the liver in fresh buffer, a much greater yield of isolated cells could be obtained. Unfortunately, this method subjected the cells to very high concentrations of digesting enzymes for prolonged periods of time, which eventually resulted in damage to the cellular membrane. Wagle and coworkers (253, 254) found that by raising the temperature of the perfusion buffer to 37°C, lower concentrations of collagenase could be used for much shorter perfusion times. This inevitably resulted in a considerable improvement in the stability of the recovered hepatocytes. Furthermore, although hyaluronidase had commonly been included along with collagenase in the enzyme mixture, Ingelbretsen and Wagle (254, 255) and others (256-258) found no evidence to support its use. In fact it was observed that at high enough concentrations, hyaluronidase was inhibitory to cell dispersion (259). Seglan (237, 256, 260) and Fry *et al*. (261) further improved the ease of tissue dispersion and the yield of intact, viable cells which retained *in vivo* metabolic characteristics by removing the intercellular Ca^{++} prior to perfusion with an enzyme buffer. The removal of Ca^{++} was accomplished either by perfusing the liver with an EDTA containing buffer, or by the efficient washout with a Ca^{++}-free buffer. An interesting aspect of the use of collagenase was revealed by Seifter and Harper (217) who reported that the presence of Ca^{++} was required for enzymic activity, which necessitated its re-addition once the endogenous Ca^{++} has been depleted. Apparently the removal of Ca^{++} during pre-perfusion creates changes in the intercellular integrity of tissue which is not reaily reversed when Ca^{++} is re-added (260). In contrast to the majority of studies which have included Ca^{++} in the collagenase buffer, Zahlten and Stratman (258) claimed no need for it. This was supported by Gallop et al. (262) who noted that collagenase normally contains a small amount of tightly bound calcium ions which are sufficient to allow enzymic activity. Furthermore, Berry (263) suggested that variations in endogenous Ca^{++} content may be responsible for reported differences in digestive action between batches of collagenase, rather than due to their inherent enzyme activity.

The development of the two-step perfusion procedure of Ca^{++} removal followed by the addition of Ca^{++} and collagenase has eliminated much of the difficulty in preparing isolated cells and resulted in the highest possible recovery of viable cells. In some instances, 55-65% w/w of the liver was recovered as isolated intact hepatocytes (253, 256, 264, 265) and reported viabilities of greater than 95% were not uncommon (253, 256, 264, 266, 267). A comparison of cell yields by utilizing various methods of isolating adult rat hepatocytes is summarized in Table 3.

3.3.2.2 Fetal and Neonatal Hepatocytes

Although general methods involving enzymic perfusion of the liver in situ undoubtedly result in the highest possible

TABLE 3. Comparison of the Efficiency of Various Tissue Dissociation Methods used in the Isolation of Intact, Viable Hepatocytes.

METHOD OF ISOLATING CELLS	TOTAL CELLS RECOVERED ($\times 10^6$)	VIABILITY (%)	NUMBER VIABLE CELLS ($\times 10^6$)	REF.
MECHANICAL DISRUPTION	10	0	0	(237)
INCUBATION OF TISSUE SLICES (followed by mechanical disruption)				
-with TPB	34.	0	0	(237)
-with collagenase/hyaluronidase	.5	60	.3	(325)
	5.	93	4.65	(250)
-with collagenase/hyaluronidase with prior Ca++ removal	29.	75	22	(588)
	20.	85	17	(325)
ISOLATED ORGAN PERFUSION				
-with citrate	21.	0	0	(261)
-with TPB	12.	0	0	(261)
-with .25% trypsin	1	0	0	(261)
-with .10% pangestion	1	0	0	(261)
-with .25% pronase	1	0	0	(261)
-with .10% collagenase	345.	55	190	(288)
	140.	100	140	(253)
-with collagenase with prior Ca++ removal	85.	95	80.7	(254)
-with collagenase/hyaluronidase	14.5	84	12.5	(326)
-with collagenase/hyaluronidase with prior Ca++ removal	136.	94	128	(256)
	76.	85	65	(588)
	121.	86	104	(265)
	30.	96	29	(267)
	100.	90	90	(264)
	138.	100	138	(253)

yield of viable cells (Table 3), there are obvious difficulties associated with this procedure when the liver is too small or only a portion of tissue is available (i.e. liver biopsy). Methods for the isolation of embryonic and neonatal hepatocytes have changed little since Moscona (268) reported the preparation of a liver cell suspension obtained from sixteen day old mouse embryos by the use of enzymic digestion. Fragments of fetal liver were incubated in the presence of a trypsin solution and the tissue dispersed into single cells by repeatedly passing them through the tip of a fine pipette. The use of trypsin has since been used extensively by a number of investigators. Nebert and Gelboin (269, 270) prepared short-term cultures from fetal hamster cells and Idoine et al. (271) and Bausher and Schaeffer (272) were able to establish long-term cultures of neonatal hepatocytes from rats less than seven days old. Similarly, Bissell and Tilles (273) investigated the morphology and function of fetal liver cells obtained from human embryos and maintained as monolayer cultures. Tsiquage et al. (274) using a modified trypsin/EDTA buffer were also able to establish long-term cultures of human fetal hepatocytes obtained from embryos of 10-23 week gestational age.

In addition, several studies have utilized the method reported by Howard et al. (249, 250) to isolate hepatocytes from fetal liver tissue. Using a solution of collagenase to dissociate cells from the livers of 19-21 day old rat embryos, Leffert and coworkers (275-279) were able to prepare differentiated fetal hepatocytes in a short-term monolayer culture. More recently, Guguen-Guillouzo _et al_. (280) and Schaeffer and Kessler (281) employed a similar procedure to culture suspensions of human liver cells obtained from fetal hepatic tissue. Acosta _et al_. (282) relied on a mixture of collagenase and hyaluronidase in solution to isolate hepatocytes from postnatal rat liver.

Besides employing either trypsin or collagenase as the digesting enzyme, the use of a neutral bacterial protease has also been reported. Nau _et al_. (283) and Takaoka _et al_. (284) isolated hepatocytes of human fetuses by digesting liver slices with Dispase 1 and found the method comparable to those which used the other enzymes. Similar results were obtained by Nau _et al_. (285) who used an EGTA/Dipase 1 combination for their tissue dispersion.

In general, although enzymic digestion of fetal liver fragments will result in the recovery of intact viable hepatocytes, yields are unlikely to be above 5% of the tissue regardless of the enzyme used. Nevertheless, this accounted for a considerable improvement over previous non-enzymic methods which have consistently given insignificant yields of intact cells.

3.3.3 Isolated Hepatocytes in Culture

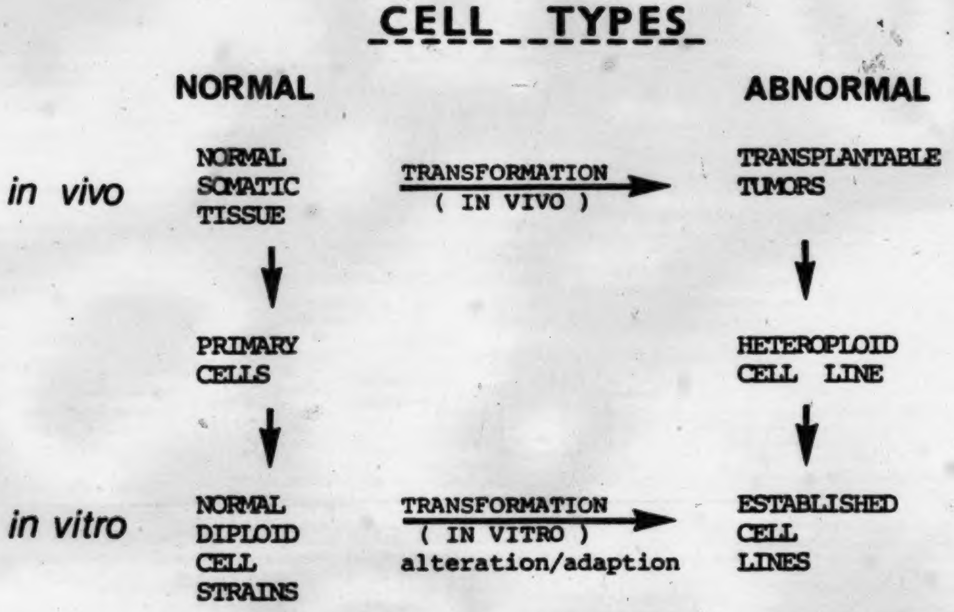
3.3.3.1 State of the Art

In 1913, Carrel (286) was the first to demonstrate that the survival of mammalian cells could be prolonged for up to several weeks in an artificial medium. Using suspension cultures containing serum, he reported that in vitro growth of connective cells isolated from various tissue fragments could be rapidly accelerated by the addition of extracts from fetal tissue. Since that time a great deal of information has accumulated concerning the growth requirements of isolated cells in vitro. The capacity of most animal cells to proliferate in culture is influenced by the characteristics of the population as a whole, such as the presence of serum and various nutrients (198, 265, 286-294), by the pH of the culture media (30, 291, 294), and the cell density (291, 295,296). However, an absolute requirement for normal growth in vitro is attachment of the cells to a solid support; thus mammalian cells are termed anchorage dependent (204, 265, 294, 297 - 299). Folkman and coworkers (300, 301) proposed a fundamental concept of growth control to explain anchorage dependency; namely, that the appropriate cell shape is critical for mitotic activity. Mere attachment of the rounded cells to a support is not enough. They must firmly adhere and spread out over the surface before they can initiate DNA synthesis (294, 297). Indeed, there is evidence which indicates that cell adhesion is involved not only in the regulation of cell growth, but in all aspects of cell survival, including morphogenesis, junction formation, differentiation, motility and malignancy (294, 297, 299, 302, 303). The exact mechanism by which cell shape is transduced to a controlling signal for regulation is still unclear. Available information does not differentiate whether cell shape actually controls cell proliferation, or whether both parameters are dependent on other factors.

While the ability of mammalian cells to multiply even though unattached to any solid support is also well known, this phenomenon is usually associated with gross morphological changes in the cell and loss of cellular function (192, 201-205, 208). This is supported by the fact that cells which demonstrate excellent growth in suspension cultures comprise such abnormal types as transformed and neoplastic cells (201, 202, 207, 221, 304, 305). The relationship between abnormal and normal cell growth in vivo and in vitro is illustrated in Figure 15 (306).

Besides anchorage dependency, monolayer cultures of primary and normal diploid cells are characterized by their morphological and chromosomal similarities to the tissue from which they are derived. Furthermore, various studies have established that normal mammalian cell strains do have a limited lifespan in vitro (202, 206, 207, 307) as depicted in Figure 16 (306). In contrast to normal strains, established cell lines have properties which resemble cancerous cells;

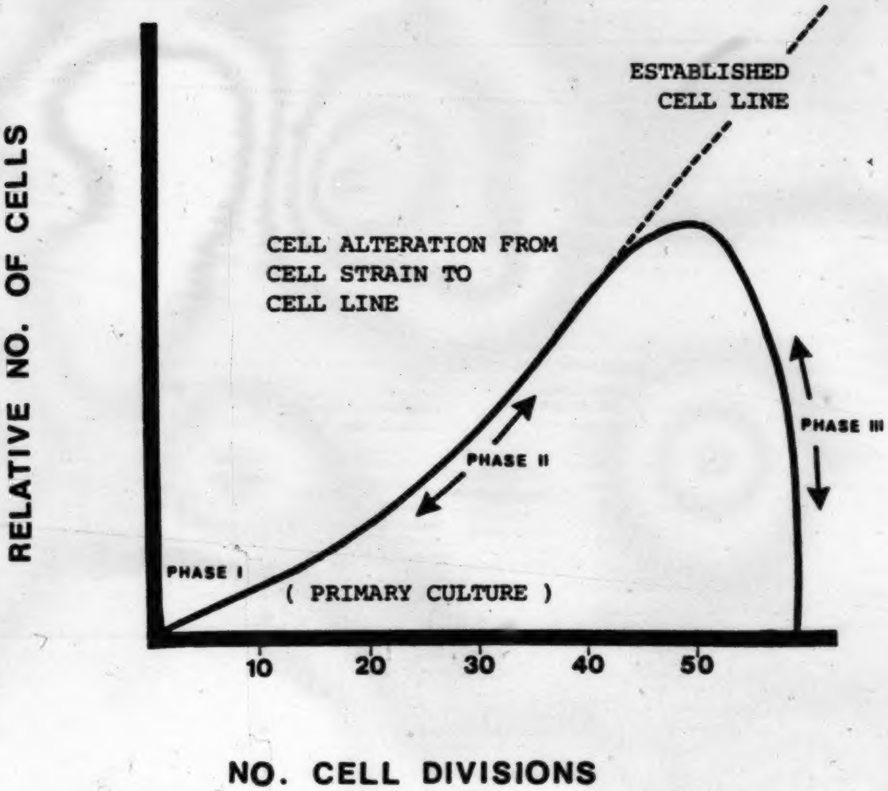
FIGURE 15. Relationship between normal and abnormal mammalian cells *in vivo* and *in vitro*. [Adapted from Hayflick and Moorhead (307)].


CELL TYPES
NORMAL
ABNORMAL
in vivo
NORMAL SOMATIC TISSUE
TRANSFORMATION (IN VIVO)
TRANSPLANTABLE TUMORS
PRIMARY CELLS
HETEROPLOID CELL LINE
in vitro
NORMAL DIPLOID CELL STRAINS
TRANSFORMATION (IN VITRO) alteration/adaption
ESTABLISHED CELL LINES

CHARACTERISTICS

DIPLOID	ABNORMAL CHROMOSOME NUMBER
NORMAL (HISTOLOGICALLY)	CANCER CELLS (HISTOLOGICALLY)
FINITE GROWTH PATTERN (50 ± 10 DIVISIONS)	INDEFINITE GROWTH PATTERN
ANCHORAGE-DEPENDENT	NOT USUALLY ANCHORAGE DEPENDENT
REQUIRES MONOLAYER FORMATION	CAN BE GROWN IN FREE SUSPENSION CULTURE

FIGURE 16. Development of cell strains and cell lines *in vitro*. [Adapted from Hayflick and Moorhead (307)].



PHASE I PRIMARY CULTURE- CELLS OBTAINED DIRECTLY FROM THE ORIGINAL TISSUE AND CULTURED AS MONOLAYERS.

PHASE II REPLICATING CELLS- MULTIPLE SUBCULTURES OF MAINTAINED NORMAL DIPLOID CELL STRAIN IN MONOLAYER CULTURES. ALTERATION OF CELLS CAN TAKE PLACE ANYTIME DURING PHASE II TO GIVE RISE TO AN ESTABLISHED CELL LINE.

PHASE III- CONTINUED SUBCULTURE OF CELL STRAINS BEYOND 50 ± 10 DIVISIONS RESULTS IN PHASE III, AND TERMINATION OF GROWTH OF THE CELLS.

that is, abnormal chromosomal numbers, altered cellular structure, an indefinite lifespan, and do not demonstrate the same anchorage dependency as normal cells. This has been observed in the recent development of many stable cell lines derived from tumors and normal cells transformed by viruses, in which growth can occur while in suspension (201, 202, 204). These cells are termed anchorage-independent (204, 306, 308) and rarely resemble the tissue of origin.

Since Schneider and Potter (211) first prepared suspensions of crudely isolated rat liver cells, considerable interest has been directed towards their use as a system in which to study liver function, while maintaining a more rigid control over many of the variables which influence activity. But despite numerous advances in culture methodolgy, the long-term maintenance of normally functional hepatocytes has remained essentially unsuccessful. In the adult liver virtually all the parenchymal cells are proliferationally inactive. Considered as reverting post-mitotic cells, that is cells with a potential dividing capacity triggered only after a special stimulus, they have a low mitotic incidence of only about 0.01% under normal physiological conditions (309, 310). Although it has been demonstrated that proliferation can be re-started under certain conditions involving partial hepatectomy (311, 312), there is little information available on this phenomenom. Only during the very early stages of development does the liver contain large populations of mitotically active cells. Consequently, nearly all hepatocytes live for as long as the animal does after they have undergone differentiation (313). Despite a prolonged lifespan in vivo, their major problem with hepatocytes in vitro is their short viability and ensuing metabolic instability. It is unfortunate that long-term cell lines cannot be considered as adequate alternatives. These cells lose most of the metabolic functions of normal adult hepatocytes by dedifferentiation.

3.3.3.2 Suspension Cultures

Cells in suspension differ from cells attached to a solid surface as a monolayer culture in a variety of ways, any of which might be concerned in the regulation of cell growth. Of particular importance is the fact that suspensions of isolated hepatocytes display severely diminished metabolic activity which may persist for just three to four hours in vitro (250). Several of the earlier biochemical studies reported a poor viability of liver cell suspensions due to the leakage of important enzymes from dispersed cells (189, 242, 245, 247). Because leakage of cytoplasmic enzymes occurs only after severe irreversible damage to the cells (246), the difficulties encountered in earlier investigations appeared to be a direct consequence of the mechanical methods of dispersing the cells. In contrast, using enzymically dissociated hepatocytes, Howard and Pesch (250) reported a substantially slower leakage of cellular enzymes and Berg et al (314) observed no leakage over at least a seven hour period after isolating cells using a collagenase/ hyaluronidase liver perfusion method. Subsequently, Haung and Ebner (235) compared enzyme induction in cells isolated by a variety of methods and found that the cells produced by enzymic procedures were superior to those obtained by a combination mechanical and chemical means. This agreed with numerous other authors who found that protein synthesis was far more efficient in enzymically prepared hepatocytes than by other methods (201, 231, 315-318). But, despite the improved integrity of enzymically derived cells, survival of functionally active cells in suspension was in most cases, still limited to just a few hours, though there have been reported exceptions. Jeejeebhoy et al. (319) using a suspension technique supplemented with horse serum, observed that hepatocytes were maintained in a viable state over a forty-eight hour period, whereas Gerschenson and Casanello (216, 320) claimed a survival time of over thirty days. In the latter instance TPB—dissociated cells were used and a full recovery of the cells after eighteen hours in suspension was reported. However, most current studies claim a viability of only three to six hours, which severly limits their use for any studies other than extremely short-term ones.

3.3.3.3 Monolayer Cultures

3.3.3.3.1 Non-proliferating Primary Cultures

Bauer *et al.* (246) stated that all cells will inevitably undergo a certain degree of trauma regardless of the isolation procedure. But whereas hepatocytes in suspension are incapable of recovery, cells cultured as a monolayer, display some ability to recover morphological integrity. Thus in some circumstances, monolayer culture techniques offer distinct advantages over cell suspensions by virtue of their much longer useful time period.

As with most techniques in cell culture, early attempts to culture highly differentiated cells usually resulted in a rapid overgrowth by more primitive cells which usually grow out as fibroblasts or as a simple endothelium(198, 199, 210, 222, 223, 321-324). In many cases, characteristics of the differentiated epithelial cells in question could be demonstrated for short periods of time before the primary cultures became overgrown by non-differentiated cells. The problem of overgrowth was eventually solved by purification of the parenchymal cells prior to culturing. This is usually accomplished by very low speed centrifugation, or more recently by gradient sedimentation (187, 255, 290, 325-327). The behavior of purified cells *in vitro* demonstrated a better correlation to *in vivo* functional characteristics than did culture from crude cell preparations.

The preparation of non-proliferating adult parenchymal rat liver cells in primary monolayer culture is now fairly routine in many laboratories, although most liver—specific functions in culture are generally reduced in comparison with what is observed *in vivo* (193-196, 209, 290, 319, 326). Primary cultures do exhibit functionally active hepatocytes during the first several days, but the activity decreases considerably by the fourth or fifth day (193, 197-200, 267, 328). Thus, although cells can remain structurally intact for up to three weeks, enzymic activity is practically negligible after just one week.

It has not been determined whether this inevitable decrease results from damage inflicted during isolation, from inadequacy of the *in vitro* environment, or as a consequence of the intrinsic nature of hepatocytes (dedifferentiation). There is increasing evidence to indicate that impairment of cellular function is a natural aging progression of cells incapable of mitotic activity (329).

3.3.3.3.1.1 Growth Requirements

Many variables combine to determine whether or not cells remain alive *in vitro* and the extent of survival can be significantly influenced by even small variations in the culture media. Ham and Mckeeham (291) outlined certain cellular growth requirements as,

i. the medium must supply all essential nutrients,

ii. physiological parameters such as temperature, pH, and osmolarity must be kept within acceptable limits,

iii. the culture system must be free from toxic or inhibitory effects, including those due to excessive amounts of essential components,

iv. precise quantitative adjustments, including balanced relationships among the components of the culture system are required, and

v. serum, which is frequently added to defined media to stimulate growth, interacts with virtually every other variable in the culture system. It also serves as a source of macromolecular growth factors that are essential for multiplication of many, but not all, types of cells.

The effects of different culture media and serum concentrations on cell survival have been extensively reviewed (198, 287-289, 291-293). The major purpose accomplished by the addition of serum has been to supply needed hormones, vitamins, amino acids, trace elements, various small molecules and essential macromolecules. Studies have also demonstrated that serum proteins play a significant role in cell attachment (291, 329) as well as a protective effect on the cells (291, 330).

It is generally accepted that all normal cells and most transformed cells require factors found in serum to survive in culture (197, 287). Most attempts to maintain hepatocytes in a serum-free medium have proved unsuccessful (331) although Bissell and coworkers (197, 332, 333) reported that functionally active parenchymal cells from adult rat liver could be maintained for up to six days provided the donor animal was previously subjected to a partial hepatectomy. In direct contrast, Bonney et al. (198) using the identical procedure, argued that serum was indeed required for proper establishment of a monolayer culture.

Significant improvements in hepatocyte culture systems have been made by the introduction of hormones into the media (287, 334-339). Most investigators agree that the addition of various hormones such as insulin (198, 288, 336, 340-342) or glucocorticoids (335, 336, 339, 340, 343, 344) will enhance the attachment efficiency of liver cells and improve cellular morphology as well as induce both enzymic activity and protein synthesis. But whereas numerous authors have reported an increase in cell survival time with the use of hormones (198, 335, 341, 344), an equal number of studies could not demonstrate a direct effect on cell viability (288, 336, 339, 340, 343).

3.3.3.3.1.2 Growth Supports

It has long been realized that certain components of the basement membrane contributed greatly to the survival of normal cells (308, 345). In order to facilitate the anchorage requirements of plated hepatocytes, various substrates made up, at least in part, of these basement components are used to support the attachment and spreading of primary cell cultures. The most common method for culturing primary liver cells has been on plastic petri dishes coated with a thin layer of collagen (198, 308, 340, 346-350) or more recently, of fibronectin (298, 351). But various improvements in the attachment matrices have reportedly resulted in a considerable increase in culture longevity. Johannson (298) described the use of petri dishes coated with laminin as the attachment protein and found it superior to either fibronectin or collagen in mediating the anchorage and spreading of hepatocytes. Sirica et al. (352) modified the procedure by culturing cells on a nylon mesh coated with a thin collagen layer. In this manner they were able to observe extended enzymic activity compared to hepatocytes maintained under similar conditions attached to collagen coated plastic surfaces. By using a floating collagen membrane, Michalopoulos and coworkers (339, 346, 353) were able to maintain normal functional liver cells for periods of twenty days or longer. They suggested the improvement over collagen coated plates was due to the fact that the shrinking membrane satisfied the requirement of parenchymal liver cells to anchor themselves to a solid surface while at the same time allowing them to aggregate without separating from the matrix.

A major advance in prolonging cell survival was made recently by Rojkind, Reid and coworkers (192, 354) who introduced the use of a complex mixture of collagens, noncollagenous proteins and carbohydrates as the attachment substrate. Called "biomatrix", it contained a significant portion of the in vivo extracellular matrix (basement membrane and ground substances) found normally in liver tissue. They demonstrated that hepatocytes which do not normally survive for more than a couple of weeks on collagen gels can survive for more than five months when cultured on biomatrix. The cells cultured were shown to have increased attachment efficiency, prolonged survival times, and that they retained several hepatocyte-specific functions.

An interesting development which has proven beneficial for maintaining epithelial cells in vitro has been to cocultivate them with feeder cells, which are usually mutagenic in nature (355-359). Using this approach, Langenbach et al. (360) seeded primary hepatocytes on a layer of transformed cells already in monolayer culture. It was demonstrated that these feeder cells significantly prolonged the survival time of the liver parenchymal cells with retention of normal biochemical and morphological characteristics. Although the mechanism by which the feeder cells facilitate maintenance of the hepatocytes is unknown, detachment of the cells and loss of enzymic activity occurred more slowly than with cells cultured on plastic.

3.3.3.3.2 Long-term, Proliferating Hepatocyte Cultures

Numerous investigations have resulted in the establishment of long-term epithelial cultures from normal (194, 195, 199, 232, 323, 359, 361-364) and regenerating (197, 363, 365-367) adult rat liver, from rat hepatomas (199, 221, 289, 362, 363) and also from neonatal (221, 222, 279, 321, 361, 368 - 374) and fetal (206, 277-279, 365, 368) rat liver tissue. In addition, established cell lines have been obtained from human fetal (224, 280, 375) and adult (376, 377) liver tissue. The principle disadvantage to long-term cultures though, is that they display few properties of normal hepatocytes *in vivo*. It is not clear whether this is an inevitable consequence of continuous proliferation or is due to a physiological inhibition caused by the culture conditions. There is evidence that the morphogenesis of cultured liver parenchymal cells is affected by the physical (372) and nutritional (202, 203, 287, 289, 304) culture conditions. Nevertheless, the majority of authors report at least some functions in common with normal adult rat liver parenchymal cells. These include induction of tyramine aminotransferase (221, 232, 304, 378) and other enzymes (221,224, 372), synthesis of albumin (197, 319, 375,378), cholesterol (379), and glycogen (197, 375), and retention of certain cytological characteristics (194, 197, 321, 322, 367, 372). Nevertheless, the morphology of cell lines especially those derived from adult liver, must be considered as uncertain. In instances where rapidly proliferating cells are found in hepatocyte cultures, they may not be parenchymal cells at all (198, 199, 222, 322, 323). The indication that a liver cell line originated from mature hepatocytes based on the premise of a few hepatocyte-like properties is not always true. Grisham (380) reported that the reason why mitotically active cells in long-term cultures express only weak hepatocyte functions is that the cell lines were probably derived from hepatic stem cells and not the mature parenchymal cells.

Because embryonic and neonatal tissue contain a higher proportion of replicating cells than mature liver, hepatocytes obtained from the former tissues are generally much easier to culture. However, they do not provide an appropriate model when differentiated functions are required, since fetal cells differ in many specialized characteristics from mature hepatocytes (381). There is no evidence at this time to indicate that immature liver parenchymal cells in culture are capable of developing into normal mature hepatocytes.

3.3.4 The Metabolism of Xenobiotics

3.3.4.1 Phase I Reactions

For the reasons outlined earlier, it has become increasingly attractive to employ isolated hepatocytes as an in vitro model for xenobiotic metabolism studies. Since Henderson and DeWaide (224) first demonstrated the capability of citrate-dissociated rat liver cells to biotransform several compounds, subsequent investigations have indicated that virtually all metabolic reactions observed in the intact animal can be demonstrated using isolated liver parenchymal cells. Both short term suspensions of freshly isolated cells and primary monolayer cultures have been used to investigate the metabolism of a wide variety of substrate models. A summary of the recorded metabolic pathways is provided in Table 4.

By far the majority of investigations involve reactions catalyzed by the mixed function oxidase (MFO) system. It has been demonstrated that the metabolic rates observed in hepatocyte incubations are usually linear for much longer periods of time than are similar incubations performed with microsomal fractions (257, 373, 382-385). However, one prominent feature that limits the activity of adult rat hepatocytes in an artificial environment is the rapid decline of cytochrome P_{450} levels. After 24-48 hours in culture, hepatocytes obtained from normal (386-389), as well as regenerating (332, 390, 391) adult rat liver retained only 10-35% of the micro-

TABLE 4. Phase I Metabolism of Various Xenobiotic Substrates in Cultured Hepatocytes.

PATHWAY	SUBSTRATE	TYPE OF CULTURE[1]	SPECIES[2]	(REF.)
AROMATIC HYDROXYLATION				
	Benzo(α)pyrene	S	R	257, 416, 417, 420
	Biphenyl	S	R	7, 136, 421, 422
	Aniline	S	R	224
		M	R	397
	Butamoxane	S	R	6
	Diphenylhydantoin	S	R	423
	Quinine	S	R	8
N-DEALKYLATION				
	Aminopyrine	M	R	402
		S	R	224
	Antipyrine	S	R	326
	Acetylmethadol	S	R	6
	Propoxyphene	S	R	6
	ρ-Chloro-N-methylaniline	M	R,M	424
	Prazepam	S	Mn	283
	Ethylmorphine	S	R	417, 423
O-DEALKYLATION				
	8-Methoxybutamoxane	S	R	6
	Phenacetin	S	R	426
	ρ-Nitroanisole	M	R	390
		S	R	425
	7-Ethoxycoumarin	S	R	3, 257
ALIPHATIC HYDROXYLATION				
	Ethinimate	S	R	6
	Barbiturate	S	R	413
	Prazepam	M	Mn	285
	Alprenolol	S	R	418
	Nicotine	M	Mk	427
ALCOHOL OXIDATION				
	Ethinimate	S	R	6
	Ethanol	S	R	428
	ρ-Hydroxynicotine	M	Mk	427
ALDEHYDE OXIDATION	Acetaldehyde	S	R	428
KETONE REDUCTION	Nabilone	S	R	413, 429
N-HYDROXYLATION	2-Acetylaminofluorene	M	R	309, 415
N-DEALKYLATION	2-Acetylaminofluorene	M	R	309
EPOXIDATION	Bromobenzene	S	R	419
HYDROLYSIS	Digitoxin	S	R	414

[1] S = SUSPENSION CULTURE, M = MONOLAYER CULTURE

[2] R = RAT, M = MOUSE, Mn = MAN, Mk = MONKEY

somal activity measured in freshly isolated cells. In most instances this marked loss of cytochrome P_{450} could not be prevented, despite the use of various media preparations (332, 386-388, 390, 391) supplemented with either serum or insulin (332, 387, 390, 392-394). However, several authors have reported this rapid decrease could at least be slowed by supplementation with various agents known to increase P_{450} levels in vivo including ascorbic acid (391), nicotinamide (388, 395), cycloheximide (396), phenobarbital (7, 392, 394, 397), and 3-methylcholanthrene (394). In addition, Decad et al. (334) had observed that higher levels of P_{450} could be maintained in the presence of testosterone, and Guzelian et al. (393) noted similar results with the addition of dexamethasone. Hydrocortisone was also thought to prevent the degradation of cytochrome P_{450}; however, Michalopoulos et al. (386) found that this hormone in fact stimulated the appearance of P_{448} and not P_{450}. Decad et al. (334) were able to demonstrate the maintenance of near in vivo levels of cytochrome P_{450} in adult hepatocytes cultured for up to twenty-four hours in a defined medium supplemented with a mixture of several peptides and steroidal hormones. Although it has been demonstrated that several hepatic functions can be retained by hepatocytes in monolayer culture for relatively prolonged periods of time (282, 304, 352-354, 368, 380) obviously cytochrome P_{450} is not included. This appears to be a reflection of the cells incompatability between long-term survival in vitro and retaining their differentiated state.

Replicating cell lines derived from hepatocytes make poor models for in vivo hepatic drug metabolism, as they fail to exhibit most properties of the drug metabolizing system, such as those associated with cytochrome P_{450} (197, 219, 334, 387). This loss of microsomal activity does not appear to be the result of continuous cell division, but probably occurs very early during the primary culture phase.

In spite of the comparable ease with which embryonic and neonatal rat hepatocytes can be cultured, their use in drug metabolism studies appeared to be of limited value. Some success has been reported with the use of fetal human liver cells (283) and primary cultures of chick embryo hepatocytes, but in general, studies have reported that levels of most enzymes responsible for xenobiotic metabolism are very low in the fetal and neonatal state (399-401). Because hepatocytes do not undergo differentiation in vitro, development of an adequate metabolism system in cultured immature liver cells is unlikely and such cells may be a poor model for the study of metabolic mechanisms which are demonstrated by more specialized adult hepatocytes. Several investigators have reported the induction of aryl hydrocarbon hydroxylase (AHH) in fetal cultures by a variety of chemical agents (219, 402, 403), but this increase was associated with the cytochrome P_{448} system (402). In fact, Owens and Nebert (402) have further claimed that the microsomal enzyme found in all liver cell lines and cultures of fetal hepatocytes is the P_{448} form and not the P_{450} protein present in more mature tissue. These results demonstrated that standard procedures for culturing hepatocytes caused significant alterations in the drug metabolizing system which must be regulated before more detailed comparison of cultures with the original tissue in vivo can be made.

3.3.4.2 Phase II Conjugation Reactions

Numerous reports have indicated that in addition to phase I primary metabolic reactions, short-term cultures of freshly isolated adult hepatocytes have an efficient capability to conjugate substrates or their metabolites (Table 5). In various instances this can offer distinct advantages in xenobiotic metabolism studies over broken cell preparations. For example, using purified liver parenchymal cells, Suolinna (384) disproved an earlier claim (404) that acetylation of sulfanilamide takes place predominantly in the reticuloendothelial cells. Murphy et al. (405) reported that the different pharmacological activities demonstrated by l- and d-drobuline were due to extensive glucuronidation of the d-isomer. In addition to acetylation and glucuronidation, other frequently observed phase II reactions include sulphate and glutathione conjugations (Table 5). As well, isolated

TABLE 5. Phase II Metabolism of Various Xenobiotic Substrates in Cultured Hepatocytes.

PATHWAY	SUBSTRATE	TYPE OF CULTURE[1]	SPECIES[2]	(REF.)
GLUCURONIC ACID	Benzoic Acid	S	R	136,
	ρ-Nitrophenyl	S	R	6, 224, 431
	2-, 3-, or 4-Hydroxyphenyl	S	R	191
	Hydroxybenzo(α)pyrene	S	R	240
	7-Hydroxycoumarin	S	R	257
	Paracetamol	S	R,M	406
	Drobuline	S	D	405
	Hydroxydiphenylhydantoin	S	R	430
ACETATE	ρ-Aminobenzoic acid	S	R,Rb	383, 384
	Sulfanilamide	S	R,Rb	383, 384
	Sulfamethazine	S	R,Rb	383, 384
SULFATE	3- or 4-Hydroxybiphenyl	S	R	191
	ρ-Nitrophenyl	S	R	431
	Paracetamol	S	R,M	406
	7-Hydroxycoumarin	S	R	3, 257
	Hydroxybenzopyrene	S	R	420
GLUTATHIONE	Paracetamol	S	R,M	406
	Hydroxybenzopyrene	S	R	420, 432
	Benzo(α)anthracene	M	R	3
	Bromobenzene	S	R	411, 433
CYSTEINE	Paracetamol	S	R,M	406
GLYCINE	Benzoic acid	S	R	136
GLUTAMATE	Methotrexate	S	R	407
		M	R	408
MERCAPTURIC ACID	Benzo(α)anthracene	M	R	3

[1] S = SUSPENSION CULTURE, M = MONOLAYER CULTURE

[2] R = RAT, D = DOG, M = MOUSE, Rb = RABBIT

accounts of compounds undergoing derivatization with cysteine (406), glycine (136), glutamate (407, 408) and mercapturic acid (3), have been reported to occur in cultured hepatocytes.

In general, these phase II reactions occurred in adult rat hepatocytes without the addition of exogenous cofactors, although conjugation rates could be influenced by the regulation of certain intracellular components (191, 409-412). At present, there are no published reports which describe the monitoring of conjugating enzyme systems in isolated hepatocytes maintained over prolonged periods of time. However, Galivan (408) did demonstrate that adult rat hepatocytes retained most of their ability to form glutamate derivatives of methotrexate during the first three days in a primary culture.

3.3.4.3 Correlation Between Isolated Hepatocytes and In Vivo Results

The usefulness of isolated hepatocytes as a model of hepatic metabolism in vivo depends considerably on their ability to exhibit metabolic profiles similar to those seen in vivo. There are several instances where such isolated hepatocytes provide a pertinent model for comparison. These include the observation that barbiturate metabolism compared very favourably in adult rat liver parenchymal cells in suspension and in the intact animal (413), whereas metabolism by rat liver 9 000X*g* supernatant fraction gave no such correlation with *in vivo* results. Guzelian *et al.* (390) studied various metabolizing enzymes (*p*-nitroanisole-*O*-demethylase, aniline hydroxylase, aminopyrine-*N*-demethylase) and found similarities between the activities of hepatocytes in primary cultures and the liver *in vivo* which could not be demonstrated using hepatic microsomes. A list of other substrates which have shown a good correlation of metabolic pathways between isolated rat hepatocytes and the intact animal includes ethinamate (6), digitoxin (414), nabilone (413), butaxmoxone (6), and 2-acetylaminofluorene (415). In contrast however, it has also been possible to demonstrate examples, including acetylmethadol (6), propoxyphene (6), benzo(A)pyrene (416), ethylmorphine (417), *N*,*N*-dimethylphenoxyethylamine(6), alprenolol (418), and aniline (417), where the rates of metabolism in isolated cells compared better with broken cell preparations maintained in the presence of an NADPH-generating system than with what occurred in the intact animal. Furthermore, studies by Michalopoulos *et al.* (394) using primary hepatocyte cultures demonstrated that induction of cytochrome P_{450} by phenobarbital treatment reflected a response related more to that shown by microsomal preparations, then to the effect expected *in vivo* (6, 416, 418, 419). In addition, agents known to inhibit metabolic activity produced similar responses in both isolated hepatocytes and subcellular fractions (6, 7, 416, 418, 419).

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4. MATERIALS AND METHODS

4.1 CHEMICALS AND BIOLOGICALS

The following lists identify the chemicals, biochemicals and biological products used. Sources are given in parenthesis. The full company name and address which corresponds to the abbreviation used are given in Section 4.5.

4.1.1 Chemicals

4.1.1.1 Substrates, Reference Compounds, and Internal Standards

Allylbenzene (Aldrich)
(+)-Amphetamine Sulfate (SKF)
(-)-Amphetamine Sulfate (SKF)
(±)-Amphetamine HCI (SKF)
Benzaldehyde (Aldrich)
Benzyl alcohol (Aldrich)
Benzoic acid (Aldrich)
p-Chlorophenylethylamine, free base (Sigma)
Cyclohexylacetone (KL)
(±)-*p*-Hydroxyamphetamine HBr (SKF)
(±)-*p*-Hydroxynorephedrine HBr (SKF)
1-Hydroxy-1-Phenyl-2-propanone (Synth. FMP)
2-Hydroxy-1-Phenyl-1-propanone (Synth. FMP)
(±)-Norephedrine HCl (Aldrich)
Phenylacetone (P&B)

erythro-1-Phenyl-1,2-propanediol (Synth. DBP)

threo-1-Phenyl-1,2-propanediol (Synth. FMP)

1-Phenyl-1,2-propanedione (P&B)

R,S-1-Phenyl-1-propanol (Synth. FMP)

R,S-1-Phenyl-2-propanol (Synth. DBP)

Propiophenone (P&B)

4.1.1.2 Derivatizing Reagents

Acetic anhydride (MCB)

R-(-)-Menthyl chloroformate (Synth. FMP)

S-(+)-α-Methylbenzylisocyanate (Aldrich)

Pentafluoropropionic anhydride (Pierce)

Trifluoroacetic anhydride (Sigma)

All other bench chemicals and solvents were of reagent grade and used without further purification, unless otherwise stated. They were purchased from various sources.

4.1.1.3 Chemical Synthesis

The following compounds were prepared by, or in co-operation with Dr. F.M. Pasutto. Data have been included as reference material only. All compounds gave mass spectra (MS), nuclear magnetic resonance (NMR) spectra, and/or infrared (IR) spectra consistent with their structure.

4.1.1.3.1 1-Hydroxy-1-phenyl-2-propanone

This compound was prepared by the nucleophilic acylation of benzaldehyde with ethoxyvinyllithium (434). The title compound contained 6% of the isomer, 2-hydroxy-1-phenyl-1-propanone (NMR and GLC evidence) which could not be removed by TLC, column chromatography, or distillation.

4.1.1.3.2 2-Hydroxy-1-phenyl-1-propanone

This compound was prepared from 2-lithio-2-phenyl-1,3-dithiane and acetaldehyde using a method described by Bowlus and Katzenellenbogen (435). The title compound was contaminated with 8% of the isomer, 1-hydroxy-1-phenyl-2-propanone (NMR and GLC evidence) and could not be purified further.

4.1.1.3.3 R(-)-Menthyl chloroformate

The chiral derivatizing reagent, R(-)-menthyl chloroformate, was prepared according to the published method of Westley and Halpern (436). A solution of this compound in toluene (50 umol/ml) was demonstrated to be stable for several months if stored over calcium carbonate at -7°C.

4.1.1.3.4 *erythro*-1-Phenyl-1,2-propanediol

Reduction of 1-phenyl-1,2-propanedione with lithium aluminum hydride ($LiAlH_4$) in tetrahydrofuran (THF) (437) gave a yellow oil which was a 2:1 mixture of the *erythro* : *threo* diastereoisomers of the title compound (NMR evidence).

To this isomeric mixture was added a small volume of diethyl ether in which only a portion of the oil was soluble. Evaporation of the ether solution gave the title compound as a colorless crystalline solid, mp. 88-90°C from chloroform [reported 91-92.5°C (438)].

4.1.1.3.5 *threo*-1-Phenyl-1,2-propanediol

This compound was prepared from *trans*-1-phenyl-1-propene by a literature method (739), mp. 52-53.5°C from petroleum ether-benzene [reported 51-53.5°C (438)].

4.1.1.3.6 1-Phenyl-1-propanol and 1-Phenyl-2-propanol

Racemate alcohols, *R*,*S*-1-phenyl-1-propanol and *R*,*S*-1-phenyl-2-propanol were prepared by the lithium aluminum hydride reduction of the corresponding phenylalkylketones (propiophenone and phenylacetone respectively) in dry diethyl ether (437).

Alcohols, *R*,*S*-1-phenyl-1-propanol and *R*,*S*-1-phenyl-2-propanol, enriched in the *S*-isomer, were prepared by the asymmetric reduction of the corresponding phenylalkylketones (propiophenone and phenylacetone respectively) with the chiral sodium *L*-prolinate borane complex prepared according to Umino *et al*. (439). Reduction of propiophenone gave a 72/28 ratio of the *S*(-)/*R*(+) enantiomers of 1-phenyl-1-propanol [determined by polorimetric analysis; based on

$[A]_D$-32.50 [C=5.1, EtOH (440)]. Reduction of phenylacetone gave a 60/40 ratio of the S(+)/R(-) enantiomers of 1-phenyl-2-propanol [based on $[A]_D$+16.10 [C=5.6, EtOH (440)].

4.1.2 Biological Products and Biochemicals

4.1.2.1 Enzymes, Coenzymes, and Intermediates

Collagenase (MP)

D-Glucose-6-phosphate, disodium salt (Sigma)

Glucose-6-phosphate dehydrogenase, Type XV from Baker's Yeast (Sigma)

Glucose-6-phosphate dehydrogenase, Type XXIV from *Leuconostoc mesenteroides* (Sigma)

β-Glucuronidase, Type H-1 (Sigma)

p-Hydroxyphenylpyruvic acid (Aldrich)

DL-Isocitric acid, trisodium salt (Sigma)

Isocitrate dehydrogenase, Type IV from porcine heart (Sigma)

α-Ketoglutaric acid, free acid (Sigma) Nicotinamide (Aldrich)

β-Nicotinamide adenine dinucleotide, oxidized form (NAD^+) Grade III, free acid (Sigma)

β-Nicotinamide adenine dinucleotide, reduced form (NADH), Grade III, disodium salt (Sigma)

β-Nicotinamide adenine dinucleotide phosphate, oxidized form (NADP+), monosodium salt (Sigma)

β-Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), Type 1, tetrasodium salt (Sigma)

6-Phosphogluconic dehydrogenase, Type IV from Baker's Yeast (Sigma)

Pyruvic acid Type II, sodium salt (Sigma)

Pyridoxal 5-phosphate, monohydrate (Aldrich)

Sulfatase, Type H-1 (Sigma)

Trypsin, 1:250 (Gibco)

L-Tyrosine, free base (Sigma)

4.1.2.2 Defined Media and Buffers

Media (with L-Glutamine, without $NaHCO_3$):

Dulbecco's Modified Eagle Medium (DMEM), with glucose and sodium pyruvate (Gibco)

Leibovitz's L-15 Medium (Gibco)

McCoy's Modified 5A Medium (Gibco)

Medium 199, with Earle's Salts (Gibco)

Minimum Essential Medium (Eagle MEM) (Gibco)

Nutrient (HAM) Mixture F-10 (Gibco)

Nutrient (HAM) Mixture F-12 (Gibco)

Waymouth's MB751/1 Medium (Gibco)

Waymouth's MB751/1 Medium, 10x concentrate (Gibco)

Buffers:

Dulbecco's Phosphate Buffered Saline (PBS), with $CaCl_2$ (Gibco)

Hank's Balanced Salt Solution (HBSS), without $NaHCO_3$ (Gibco)

HEPES Buffer (N-2-hydroxyethylpiperazine-N-ethanesulfonic acid) sodium salt (Sigma)

Trisma Base [Tris (hydroxy methyl) amino methane]
(Sigma)

Common salts used in the preparation of buffers were of analytical grade and obtained from various sources.

4.1.2.3 Antibiotics and Serum & Serum Products

Albumin (Bovine), Fraction V (Sigma)

Benzylpenicillin (Penicillin-G), sodium salt (Sigma)

Dexamethasone (Hexadral Phosphate Inj.),
sodium phosphate (Organon)

Hydrocortisone (Solu-Cortef Inj.), sodium
succinate salt (Upjohn)

Insulin (Toronto-Insulin) from beef and
pork, zinc salt (Connaught)

Serum (Fetal Bovine) mycoplasm tested and
virus screened (Gibco)

Streptomycin, sulfate salt (Sigma)

Transferrin (Siderophillin) from human (Sigma)

4.1.3 Water

Water utilized in the preparation and maintenance of cell cultures was pyrogen-free, triple glass-distilled, demineralized water obtained with a Corning AG-11 Distillation Unit (Corning). All water was obtained at the maximum purity setting and sterilized by autoclave (125°C, 15 psi, 45 min.) prior to use.

4.2 INSTRUMENTATION

4.2.1 Gas-Liquid Chromatography

4.2.1.1 Flame Ionization Detection

Gas-liquid chromatographic (GLC) analyses incorporating flame ionization detection (FID) were conducted on a Hewlett-Packard Model 5710A gas chromatograph connected to a Model 3380A integrator. GLC packed glass columns and operating conditions are listed in Table 6. For analysis by capillary column, a 52 m SE-30 (SCOT) glass column (i.d. 0.4 mm) (SGE) was used with temperature programming from 150-250°C (4°/min.). Flow rate of the carrier gas, helium, was 3 ml/min. (17 psi.). Injection port and detector temperatures in all instances were 250°C and 300°C respectively.

4.2.1.2 Electron-Capture Detection

For electron-capture detection (EC-GLC), analyses were performed on either of two available systems. Both were equipped with a 15 mCi ^{63}Ni source linear EC-detector, and operated at injection port and detector port temperatures of 250°C and 275°C respectively.

One EC-GLC, which was used with packed columns (Table 6), was a Hewlett-Packard Model 5730A gas chromatograph (Model 3380A integrator). The carrier gas and make-up gas at the detector were both argon:methane (95:5). Flow

TABLE 6. Packed Columns used in GLC and GLC/MS Analyses.[1]

SYSTEM	LIQUID PHASE	SUPPORT	COLUMN i.d. (mm)	GAS FLOW RATE (ml/min)	COLUMN LENGTH (m)
(FID-GLC)					
A	3.8% OV-101	chromosorb 750 (80–100 mesh)	4	60	1.26
B	2.5% Carbowax 20M 2.5% KOH	chromosorb 750 (80–100 mesh)	4	60	1.26
C	5.0% OV-17	chromosorb 750 (80–100 mesh)	2	30	2.56
(ECD-GLC)					
D	5.0% OV-101	chromosorb 750 (80–100 mesh)	4	60	1.26
E	5.0% OV-17	chromosorb 750 (80–100 mesh)	4	60	1.26

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[1] Oven operating temperatures are discussed in text.

rate at the detector was the same as that through the column (Table 6).

The second EC-GLC was a Hewlett-Packard Model 5830A chromatograph (Model 18850A integrator), equipped for capillary GLC. A 20 m Carbowax 20M fused silica capillary column (Supelco) was used with temperature programming from 80-220°C (20°/min.). Carrier gas (helium) was at 7 psi and detector make-up gas, argon:methane (90:10) was at a flow rate of 36 ml/min.

4.2.2 Gas-Liquid Chromatography - Mass Spectrometry

4.2.2.1 Electron Impact Mass Spectrometry

Electron Impact Mass Spectral (EI-MS) analyses were carried out on a Hewlett-Packard quadrupole mass spectrometer Model 5981A attached to a 5934A data system. The inlet system comprised a Hewlett-Packard Model 5710 GLC equipped for packed columns. MS operating conditions were: ionization energy, 70 eV; scan speed, 100 amu/sec.; dwell time, 200 msec.; ionization source temp., 180°C; separator temp. same as GLC oven temperature. GLC columns and operating conditions were as described above for GLC analysis with packed columns.

4.2.2.2 Chemical Ionization Mass Spectrometry

Chemical Ionization Mass spectral (CI-MS) analyses were performed on a combined Hewlett-Packard Model 5840A GLC/5985 mass spectrometer with dual EI/CI sources and a Model 7920 data system. CI-MS operating conditions: electron energy, 175 eV; reactant gas, methane; ion source pressure, 0.6 Torr. GLC operating conditions used a 10 m SE-52 fused silica capillary column (Supelco) with temperature programming 80-280°C (30°C/min.). Flow rate of carrier gas, methane, was 4 ml/min.

4.2.3 Nuclear Magnetic Resonance and Infrared Spectrometry

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM 360A 60 MHz NMR spectrometer. Deuterated chloroform ($C\underline{D}Cl_3$) (Aldrich) was used as the solvent and tetramethylsilane (TMS) (Aldrich) as the reference standard.

Infrared (IR) spectra were recorded on an Unicam SP 1000 infrared spectrometer as films between NaCl plates or incorporated in KBr discs.

4.2.4 Ultraviolet and Colorimetric Spectroscopy

All ultraviolet (UV) measurements were obtained using an Unicam SP 1800 double beam UV spectrometer with linear strip chart recorder. Colorimetric measurements were performed on a Bausch & Lomb Spectronic 20 Spectrophotometer. All readings were recorded as absorbance values.

4.2.5 Optical Polarimetry

Optical rotation measurements were made using a Carl Zeiss Circular Polarimeter 0.01° equipped with a sodium D lamp (589.3 nm).

4.2.6 Thin-Layer Chromatography

In some cases where purification of substrates or of extracts from metabolic experiments was required, thin layer chromatography (TLC) was performed. Samples were usually run on TLC plates spread to a thickness of 0.25 mm with Silica Gel 60-F-254 (EM). A number of different solvent systems were utilized and compounds were detected by short-wave UV light (254 nm).

4.2.7 Microscopy and Photomicroscopy

Light microscopy of isolated cells was performed on an Olympus Inverted Microscope (Model CKC-II) with phase contrast attachment. Photomicroscopy was carried out on a Leitz Wetzlar Dialux-Pol Polarizing Microscope (with built-in light source) connected to a Leitz System Camera. Photographs were taken on Instamatic Polaroid Film (Land Film Type 107).

4.3 ANIMALS

4.3.1 General Handling

Two species of animal (rat, rabbit) and two strains of rat (Wistar, Sprague-Dawley) were used, depending on the study. Male, Wistar white rats (Woodlands Lab.) and male, New Zealand white rabbits (Vandermeer Farms) were utilized essentially for in vitro liver homogenate and in vivo urinary metabolism studies. Sprague-Dawley rats (male, female) acquired from Bioscience Animal Services were used to supply fetal, neonatal, and adult tissue for isolated cell studies. Randomly obtained rats were used early in the study, but subsequently inbred rats were utilized.

All animals were allowed food and water ad lib until required for experiments; rats were maintained in plastic cages on cedar chip bedding, and rabbits were housed on wire suspension cages. Conditions were adjusted so the animals would be subjected to days consisting of 12 hours of continuous light, followed by 12 hours of darkness.

4.3.2 Broken Cell Studies

4.3.2.1 Preparation of Cell Fractions from Liver/Brain Tissue

Adult, male animals were fasted overnight and sacrificed by cervical dislocation. Livers and/or brains were removed immediately and placed in ice-cold 1.15% (isotonic) KCl buffer. All subsequent procedures were carried out at 4°C. A 20% (w/v) tissue homogenate was prepared using a Potter homogenizer with a Teflon pestle, and centrifuged at 500X*g* for 15 minutes to remove unbroken cells, cell nuclei, and tissue debris. The tissue supernatant was prepared by centrifuging the 500X*g* homogenate at 10 000X*g* for 20 minutes and adjusting the volume of the clear fraction with 1.15% KCl solution so that 1 ml supernatant corresponded to 0.2 g of original tissue. The mitochondrial fraction was obtained by collecting the 10 000X*g* density pellet of the 500X*g* homogenate.

Portions of the 10 000X*g* supernatant were centrifuged at 105 000X*g* for 60 minutes at 4°C to obtain the microsomal fraction (105 000X*g* resuspended pellet) and cytosol fraction (105 000X*g* supernatant). Protein contents of the microsomal and cytosol fractions for standardization of enzyme activity were determined by the method of Lowry *et al*. (441) as modified by Miller (442).

4.3.2.2 Standard In Vitro Incubation Procedures

The substrates (dissolved in 0.1 ml 95% ethanol) were added to open 25 ml Erlenmyer flasks containing tissue (10 000X*g* supernatant, microsomal fraction, or cytosol fraction) corresponding to 0.2 g original liver or brain tissue and either an NADPH- or NADH-generating system (glucose-6-phosphate, 20 umol; $MgCl_2$, 20 umol; NADP+ or NAD+, 4.4 umol) in Tris-KCl buffer, pH 7.4 (total volume 6.0 ml/flask).

Mixtures were incubated at 37°C for 60 minutes in a shaking Dubnoff incubator (120 rpm) under atmosphere. Incubations were terminated with the addition of 0.2 ml of 3 N perchloric acid.

In some instances the glucose-6-phosphate dehydrogenase system described above to generate NADPH/NADH was replaced with a standard isocitrate dehydrogenase system. This involved the addition to the tissue fraction of NADP+ or NAD+ (6.0 umol); isocitrate (30 umol); $MgCl_2$ (20 umol); and $MnCl_2 \cdot 4H_2O$ (20 umol) in Tris-KCl buffer (pH 7.4) for a total volume of 6.0 ml/flask.

Any modifications made to either system are described in the text.

4.3.3 Isolated, Intact Hepatocyte Studies

4.3.3.1 Preparation of Media and Buffers

Defined media and buffers obtained in the powder form from commercial sources, or formulated during the studies, were prepared using sterile, deionized, triple distilled water, and were immediately resterilized by filtration using a 0.22 um membrane filtration unit (MP Corp.). All media were supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), and transferrin (3 ug/ml). Further modifications to the culture media are discussed in the text. Substances to be incorporated were added directly to the media or were dissolved in Hormone Solvent (Sigma) prior to addition.

Non-commercial solutions employed for the dissociation of liver tissue included the following formulations:

Buffer A: 8.3g NaCl, 0.5g KCl, 2.4g HEPES, H_2O to 1000 ml, adjusted to pH 7.4 with 1 M NaOH.

Buffer B: 8.3g NaCl, 0.5g KCl, 2.4g HEPES, 0.1g EDTA (tetrasodium salt), 0.1g EGTA (free acid), H_2O to 100 ml adjusted to pH 7.4 with 1 M NaOH.

Buffer C: 3.9g NaCl, 0.5g KCl, 0.7g $CaCl_2 \cdot 2H_2O$, 24.0g HEPES, 0.5g collagenase, H_2O to 1000 ml, adjusted to pH 7.6 with 1 M NaOH.

Buffer D: 8.0g NaCl, 0.20g KCl, 0.10g $CaCl_2 \cdot 2H_2O$, 0.10g $MgCl_2 \cdot 6H_2O$, 1.15g $Na_2HPO_4 \cdot 2H_2O$, 0.20g KH_2PO_4, 1.5g trypsin (1:250), H_2O to 1000 ml, adjusted to pH 7.4 with 1 M NaOH (733).

All dissociation buffers were equilibrated with carbogen gas (95% O_2, 5% CO_2) and warmed to 37°C prior to use.

The composition of the buffer employed as the gelling solution during the preparation of cell entrapped carrageenan beads (immobilized cells) was as follows:

Buffer E: 12.0g Tris base, 6.0g KCl, 2.4g $CaCl_2 \cdot H_2O$, H_2O to 1000 ml, adjusted to pH 7.4 (at room temperature) with 5 M HCl (approximately 20 ml). This buffer was sterilized by steam under pressure (125°C, 15 psi, 45 min.).

4.3.3.2 Preparation of Isolated Hepatocytes

4.3.3.2.1 Adult Rat Hepatocytes

Male, Sprague-Dawley rats weighing 200-250 g were fasted for 24 hours prior to isolation of liver cells. Surgery was routinely begun between 0730 and 0830h. The liver perfusion method developed by Seglen (256) for isolating hepatic cells with collagenase was used with some modifications. All procedures were carried out under strict aseptic conditions in a Laminar Flow Hood (Can. Cab.) and all equipment, buffers, and media were pre-sterilized.

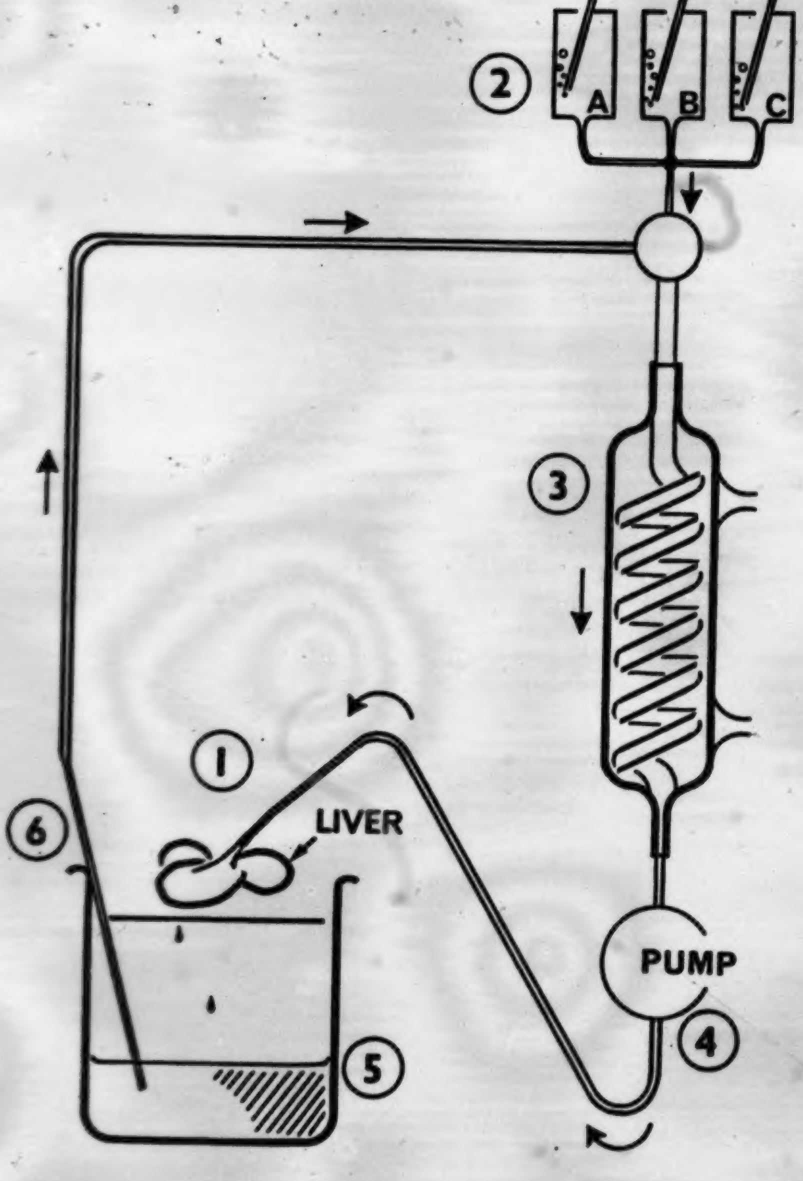
Rats were anesthetized with an *ip.* injection of sodium pentobarbital (Nembutal Sodium, Abbott, 5 mg/100g body wt.). The abdomen was opened and the portal vein (*vena porta*) exposed. The lower portion of the vein was tied off, and the upper portion cannulated with sterile IntraMedic polyethylene tubing (PE-50) (Clay Adams), which had been bevelled and rounded off.

An initial washout perfusion of the liver *in situ* (flushing without recirculation) was started immediately upon cannulation, at a flow rate of 15-20 ml/min. with the warm (38°C) Ca^{++} free buffer (Buffer A), and continued for 4-5 minutes to remove the blood from the tissue. The perfusion equipment was as shown in Fig. 17. If necessary, the liver lobes were gently manipulated in order to facilitate the complete removal of blood. It was very important that the liver tissue was uniformly perfused, and all forms of vascular occlusions were avoided. After the liver was completely blanched, a cut was made in the lower vena cava to permit free outflow, and the perfusion medium was changed to Buffer B which contained the chelating agents (.26 mM EDTA, .26 mM EGTA) (Sigma).

While being perfused, the liver was cut loose from the carcass and positioned on a stainless steel net supported in a glass beaker (with outlet). After 5-6 minutes, perfusion with Buffer B, the buffer was replaced with Buffer A (2 min.) in order to wash away the remaining EDTA/EGTA. Finally the liver was perfused with a 0.05% collagenase solution (Buffer C) at 30 ml/min. with recirculation of the perfusate. After approximately 10 minutes, the consistency of the tissue was soft enough to disintegrate on pressure, and was easily dispersed (in the collagenase buffer) with a stainless steel comb while being held at the portal region with forceps.

Combing was continued until only a white residue remained on the mesh. From then on the cells were maintained at room temperature. The detached cells were completely

Figure 17. Schematic representation of the adult liver perfusion apparatus: 1) liver, perfused through portal vein; 2) reservoir, (A) Ca++ free buffer, (B) EDTA/EGTA buffer, (C) collagenase buffer. All solutions were pre-warmed to 37°C and saturated with carbogen gas; 3) water-jacketed coiled tube maintaining perfusate temperature at 37°C; 4) peristaltic pump; 5) collection beaker with stainless steel wire net to support tissue; 6) tubing for recirculation of perfusate.



dissociated from one another by gently passing the perfusate through a 21 gauge needle. The resulting crude cell suspension was filtered through a stainless steel screen of 131 um pore size and the cells pelleted at 400 rpm for 2 minutes in 15 ml sterile, disposable, centrifuge tubes (Corning). The liver parenchymal cells were purified by resuspending the cell pellet in MEM and recollecting the cells by centrifugation at 250 rpm (5 min.). This step was repeated twice more with recovery of the cells the first time at 100 rpm (7 min.) and secondly at 60 rpm (10 min.). The last pellet containing the purified hepatocytes was finally resuspended in the medium in which they were to be seeded. Concentrations of single cells were calculated through the use of a hemacytometer. The percentage of viable hepatocytes was determined based on the ability of cells to exclude trypan blue dye following the mixture of one part cells to five parts 0.4% trypan blue (isotonic solution) (Gibco) for 5 min. at 23°C (388). The proportion of cells to incorporate the dye was then determined by light microscopy.

The time from beginning of surgery to incubation of cells was 1.5-2 hours.

4.3.3.2.2 Fetal/Neonatal Rat Liver Cells

Fetal (15-19 days pc.) or newborn (1-7 days old) inbred Sprague-Dawley rats were used for this study. Animals were decapitated and livers were aseptically removed and temporarily placed in cooled (4°C) MEM containing 10% FBS. The pooled livers were carefully minced with a scalpel blade into fragments approximately 1 mm^3 in size. The PBS was then siphoned away, and the mince was transferred into stoppered flasks containing 10 volumes of the dissociation medium which consisted of 5 parts collagenase buffer (Buffer C) and 1 part trypsin buffer (Buffer D). The mixture was incubated at 37°C for 20 minutes in a rotary shaking water-bath (150 rpm). The flask was removed and the tissue allowed to settle for 5 minutes. Supernatant from this first enzyme treatment was carefully poured off and discarded. Three further incubations were carried out using successive enzyme ratios (collagenase : trypsin) and incubation times of 5:2 (20 min.), 1:1 (15 min.) and 2:5 (10 min.). The supernatants from these three treatments were combined and filtered to remove any undigested tissue. Because of the presence of semi-digested connective tissue, the suspension medium takes on a viscous nature where the cells tended to become attached together with long mucinous strings. This necessitated dilution of the suspension with MEM in order to decrease the viscosity and permit collection of the cell pellet by centrifugation. Dilution was normally repeated five or six times before the cells could be settled out. The loosely packed cells were resuspended in the appropriate culture medium. Cell yield and viability were determined in the same manner as that employed with adult hepatocytes, and the suspension was adjusted to a final concentration of 2 x 10^6 cells/ml medium.

4.3.3.3 Standard Culture Conditions and Preparations

4.3.3.3.1 Monolayer Cultures

4.3.3.3.1.1 Preparation of Rat Tail Collagen Solution

Approximately 1 g of collagen fibres (tendons) were dissected from two rat tails and placed in sterile H_2O. Tendons were minced with sterile scissors and suspended in 300 ml of a sterile 1:1000 solution of acetic acid in H_2O. The minced tendons were soaked for 48 hr. with occasional shaking. At 48 hr. the undissolved fibres were removed by centrifugation and the clear solution stored in a sterile container at 4°C (443).

4.3.3.3.1.2 Preparation of Collagen Thin-Layer

The collagen solution described above (1.5 ml) was spread evenly in 60 mm Nunclon plastic tissue culture petri dishes (or 0.7 ml in 40 mm dishes) (Nunc.). The plates were placed in a drying oven at 70°C for 12 hours, at which time a thin-layer of collagen residue remained attached to the bottom of the plastic dish. The plates were then rinsed several times with 2 ml MEM in order to remove any remaining acetic acid.

4.3.3.3.1.3 Preparation of Collagen Gel

Collagen gels were made by heat gelation of soluble collagen at neutral pH in the following way:

The collagen solution was spread in the 60 mm culture plates as described above for the thin-layer. To this was added 0.5 ml (0.2 ml for 40 mm plates) of a 2:1 mixture consisting of 10x concentrated Waymouth's MB751/1 Medium and 0.5 N NaOH. The mixture was stirred in the culture plate for approximately 20 seconds, and placed in an incubator at 37°C in a humidified atmosphere for 1 hour. The incubation resulted in formation of a stable gel.

4.3.3.3.1.4 Preparation of Floating Collagen Membrane

Twelve hours after inoculation of the cells on to the collagen gel, the gel was loosened from around the edge of the culture dish with a sterile Pasteur pipette. The plate was gently swirled, allowing the gel to completely detach from the plastic, and float around freely in the culture medium.

4.3.3.3.1.5 Incubation Conditions

For a typical culture, 2 x 10^6 adult hepatocytes (or 5 x 10^6 immature cells) in 3 ml of medium were plated in 60mm tissue culture plates. The cultures were maintained in a humidified environment of 95% O_2 - 5% CO_2 at 37°C. After 8 hours incubation time, the medium and non-attached cells were removed and replaced with fresh, pre-warmed medium. Subsequent medium changes were made at 24 hours and every 48 hours thereafter, unless otherwise stated. The pH values of the cultures were monitored by the presence of phenol red indicator dye in the medium.

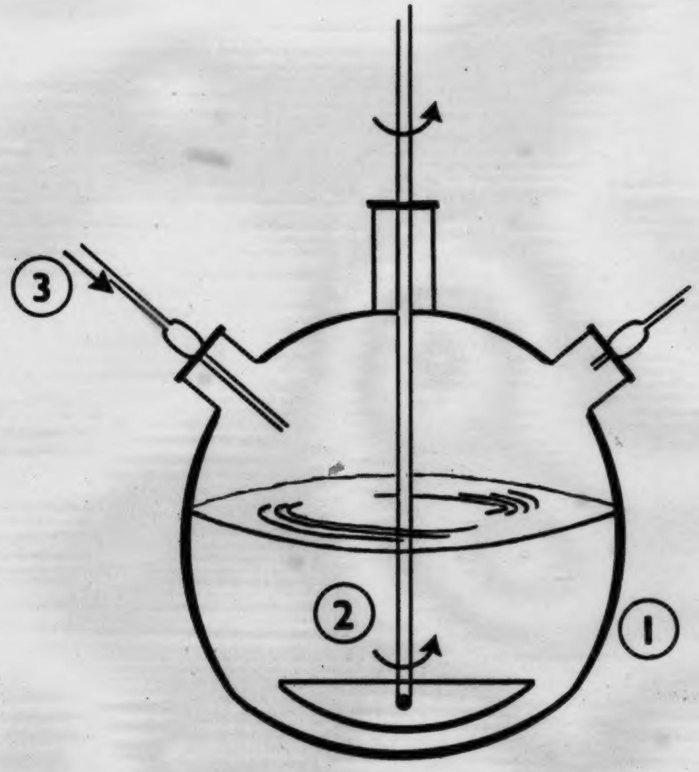
Any substrates to be added were dissolved in HBSS, sterilized by membrane filtration, and usually included along with the first change of medium.

4.3.3.3.2 Suspension Cultures

For long-term suspension cultures (greater than 12 hr), cells (mature, 10^5/ml medium; immature, 5 x 10^5/ml medium) were introduced into pre-silanized, three-necked, round bottom flasks (Fig. 18). Considerable effort was made to duplicate the proper incubation conditions of atmosphere, temperature and sterility. Because of the large volumes of medium involved (150-300 ml), a humidified atmosphere was assured. Evaporation of the medium was not a problem. Cells were kept suspended by an overhead, motor driven stirring rod.

For shorter-term suspensions (less than 8 hr.) which were used for quantitative metabolism studies, cells (mature, 5 x 10^5/ml medium; immature, 2.5 x 10^6/ml medium) were placed into pre-silanized, covered Erlenmyer flasks and incubated in a rotary shaking waterbath (60 rpm) at 37°C under a carbogen gas atmosphere. Any substrates were added

FIGURE 18. Schematic representation of the long-term suspension culture apparatus: 1) pre-silanized, three-necked round bottom flask, containing 150-300 ml medium. Temperature maintained at 37°C through use of a water bath; 2) overhead, motor driven stirring rod for suspension of cells; 3) gas inlet with sterilizing (0.22 µm) filter to maintain carbogen atmosphere.



as sterile isotonic solutions at the beginning of the incubation.

4.3.3.3.3 Immobilization Cultures

Freshly isolated adult hepatocytes were immobilized in a semi-permeable matrix in the following manner. A 4% isotonic solution of NJAL 724 carrageenan (FMC) was prepared (0.12 g NaCl, 0.4 g carrageenan, H_2O to 10 ml, adjusted to pH 7.4 with HEPES) and sterilized by autoclaving (125°C, 15 psi, 10 min.). Immediately following sterilization, in order to prevent congealing the solution was kept at 40° until used. The isolated hepatocytes, adjusted to a concentration of 25-30 x 10^6 cells/ml medium and warmed to 37°C, were mixed with twice the volume of the carrageenan solution to give a final carrageenan strength of 2.6%. Very rapidly, this mixture was added dropwise through a 23 gauge needle into a sterilized gelling solution (Tris-KCl-$CaCl_2$; Buffer E) maintained at room temperature. The carrageenan immediately gelled upon coming into contact with the metal cations (K^+, Ca^{++}) in solution and the lower temperature. The result was formation of stable, semi-permeable, bead-shaped gels, approximately 3 mm in diameter. The beads were collected and rinsed with normal saline to remove excess salts.

Typically 14-16 g of beads were produced during a single experiment containing approximately 150 x 10^6 cells

(10^7 cells/g carrageenan) Aliquots of the hepatocyte entrapped beads corresponding to about 5 x 10^6 cells were placed into 60mm culture plates along with 5 ml medium.

The complete culture media consisted of 2 parts DMEM/F-12 (1:1) medium and 1 part modified Buffer E (adjusted to pH 7.7 at RT, which equals pH 7.4 at 37°C). Medium was supplemented with 20% FBS, penicillin (100 U/ml) and transferrin (3 ug/ml). Incubation conditions were carried out in the same manner as cells seeded as monolayer cultures.

4.3.3.4 Enzyme Assay Procedures

For the measurement of cell enzyme activities, published procedures for the assay of tyrosine aminotransferase (TAT) (444) and lactate dehydrogenase (LDH) (445) were used. In studying the survival of attached cells it was found to be difficult to detach the hepatocytes from the substratum by trypsinization (446). Cells on plastic or thin collagen layer were scraped off with a rubber spatula, homogenized, and cellular debris removed by centrifugation. A small aliquot of the supernatant was used for the assays. Hepatocytes embedded into the collagen gel or floating collagen membrane were disrupted by sonification followed by homogenization while still attached to the substrate. Collagen debris was also removed by centrifugation and did not interfere with measurement of enzyme activities. Assays could also be performed on cells entrapped within the carrageenan beads by similarly separating the matrix from the medium. After disruption of the cells (sonification, homogenization), the carrageenan was removed by high speed centrifugation (3000 rpm) leaving the clear supernatant containing the enzymes for testing.

Cellular protein levels were determined in the same manner as with the tissue homogenates (441, 442).

4.4 ANALYSIS OF METABOLIC PRODUCTS

4.4.1 Ketones, Metabolites, and Related Compounds

4.4.1.1 Quantitative Analysis of Metabolites (In Vitro)

For quantitation of metabolites from in vitro incubations, an internal standard (cyclohexylacetone, 0.1 or 0.4 umol) was added to the incubation flasks just prior to extraction. Incubation mixtures were adjusted to pH 7.4 with 10% NaOH, and extracted twice with an equal volume of a distilled diethylether-methylene chloride mixture (3:2). The extracts were concentrated on a water bath (42°C) to 30 ul and analyzed by GLC.

For the quantitation of the diastereoisomers of 1-phenyl-1,2-propanediol, additional incubation mixtures to which allylbenzene (0.1 umol) was added as the internal standard were extracted as above, but were evaporated to dryness. Each metablic residue was then treated with 20 ul TFAA prior to resolution of the erythro and threo isomers by GLC.

4.4.1.2 Stereochemical Analysis of Metabolites (In Vivo/In Vitro)

4.4.1.2.1 Experimental Procedure and Extraction

The in vivo study involved the oral administration of each substrate to rats (male, Wistar, 250 g) and rabbits (male, New Zealand Whites, 2 kg). The animals were housed separately in metabolic cages and urine was collected over 48 hours and stored at 4°C until examined. Bulked urine samples from each group of treated animals were divided into three equal portions. One portion was adjusted to pH 7.4 with solid sodium bicarbonate and extracted three times with equal volumes of a distilled diethylether-methylene chloride mixture (3:2). A second portion was buffered to pH 7.0 with phosphate buffer and hydrolyzed for 36 hours at 37°C after the addition of β-glucuronidase (15 000 U/ml). The sample was then extracted as above after re-adjusting the pH to 7.4 with sodium bicarbonate. The third portion was acidified to pH < 1 with conc. HCl and autoclaved at 125°C and 15 psi for 45 minutes. After hydrolysis was complete, the pH was returned to 7.4 with 10% NaOH and the resulting solution was extracted as described above.

For the in vivo studies, each substrate (phenylacetone, propiophenone, or 1-pheny-1,2-propanedione) was incubated under standard conditions with 10 000Xg liver homogenate supernatant (rat or rabbit) fortified with either an NADPH- or an NADH-generating system. Extraction of metabolic products was carried out as described for the first portion of the in vivo study.

All extracts were concentrated and yielded yellowish oils, small aliquots of which were used for derivatization and analysis.

4.4.1.2.2 Derivatization

R(-)-Menthyloxycarbonyl derivatives were prepared as follows: 100 ul of a standard R(-)-menthyl chloroformate solution (50 umol/ml toluene) were added to a small portion of the concentrated extract (20 ul) dissolved in 100 ul pyridine. The mixture was allowed to react at room temperature for 30 minutes. After washing with water (1 ml), the organic phase was removed, dried over sodium sulfate and concentrated under nitrogen to 5 ul, of which 0.2 ul was used for GLC.

N-(1-Phenylethyl)urethanes were prepared by adding 50 ul of an S-(+)-α-methylbenzyl isocyanate solution (30 umol/ml toluene) to a portion (20 ul) of the concentrated extract dissolved in 100 ul toluene. The mixture was tightly sealed under a nitrogen atmosphere in a Reacti-vial (0.2 ml, Pierce), and kept at 120°C for 2 hours. The reaction mixture was concentrated to 5 ul under a N_2 stream and a suitable portion used for GLC analysis.

GLC resolution of the erythro and threo diasterioisomers of 1-phenyl-1,2-propanediol was accomplished by converting them to their O,O-di(trifluoroacetyl) derivatives in the same manner as reported in Section 4.4.1.1, except that an internal standard was not added to the biological sample.

4.4.2 Trace Metabolic Amines

4.4.2.1 Rat Brain Metabolism

4.4.2.1.1 Experimental Procedure (*In Vivo*/*In Vitro*) and Preparation of Biological Samples

Male Sprague-Dawley rats (150 ± 20 g) were used. For the *in vivo* studies, rats were given a single injection (*ip*.) of 74 umol of each substrate, amphetamine HCl, *p*-hydroxyamphetamine HBr, norephedrine HCl, or *p*-hydroxynorephedrine HBr, (10.0, 11.19, 11.19, and 12.37 mg/kg respectively, calculated as the free base). Compounds were administered in normal saline. Control animals were concurrently injected (*ip*.) with 0.2 ml saline. All animals were sacrificed exactly 60 minutes after treatment. The complete brain was rapidly removed and homogenized in three volumes of cold 0.4 N perchloric acid and centrifuged at 10 000X*g* for 15 minutes. Supernatant equal to 1.0 g whole brain was used in the analysis.

For the *in vitro* studies, substrates (1.0 umol) were incubated under standard conditions in the presence of 10 000X*g* rat brain supernatant (0.5 g original tissue) fortified with an NADPH-generating system (modified volume 3.8 ml). Incubations were terminated after 60 minutes by the addition of 0.2 ml of 2 N perchloric acid.

4.4.2.1.2 Extraction and Derivatization

Tissue samples [*in vivo* brain supernatant (4 ml) or *in vitro* incubation mixture (4 ml)], to which 1.0 ug of *p*-chlorophenylethylamine was added as internal standard, were adjusted to pH 7.8 by the addition of solid $KHCO_3$. After all effervescence had ceased, 500 ul sodium phosphate buffer (0.1 m, pH 7.8) was added to each sample, which were then centrifuged (2 000X*g*) to remove any precipitate (protein, potassium perchlorate) formed. The supernatant was removed and agitated (1.0 min.) with 5.0 ml of a 2.5% solution of di(2-ethylhexyl)phosphoric acid (DEHPA) (Sigma) in chloroform. Following separation of the two phases by centrifugation (5 min.; 1 000X*g*), the aqueous layer was discarded and the chloroform layer back-extracted with 3.0 ml of .5 N HCl. The aqueous layer was retained and basified with solid $NaHCO_3$.

Procedure A - For the analysis of amphetamine and norephedrine, the basified solution was adjusted to pH 9.0 with 10% NaOH and extracted with 4.0 ml of ethyl acetate and the organic layer was evaporated to dryness under a stream of nitrogen. The residue was perfluoroacylated and treated further as described below.

Procedure B - For the analysis of *p*-hydroxyamphetamine and *p*-hydroxynorephedrine, acetic anhydride (300 ul) was added to the basified solution followed by small additional quantities of $NaHCO_3$ with shaking until all effervescence ceased. Ethyl acetate (4.0 ml) was added to the aqueous phase and the mixture shaken for 2 minutes. To the separated organic layer, 300 ul of 10 N NH_4OH was added and the mixture vortexed for 30 minutes. Addition of 300 ul of 6 N HCl was followed by shaking (20 sec.) and centrifugation. The ethyl acetate layer was removed and evaporated to dryness under N_2.

The residues obtained from Procedures A and B above were separately dissolved in 35 ul ethyl acetate. To this was added 75 ul of either pentafluoropropionic anhydride (PFPA) or trifluoroacetic anhydride (TFAA), and the ensuing reaction was allowed to proceed at 85°C (60 min.) or RT (30 min.), respectively. Each mixture was evaporated to dryness, and the residue was partitioned between toluene (200 ul) and 0.1 M sodium phosphate buffer (200 ul; pH 7.4). Each organic layer was retained for EC-GLC and GLC-MS analysis.

4.4.2.2 Isolated Hepatocyte Metabolites

Cell cultures being analyzed for p-hydroxyamphetamine as a metabolite of amphetamine (1.0 umol) were prepared in the same manner as they were for enzyme assays (Section 4.3.3.4). Prior to actual analysis using assay Procedure B as described in Section 4.4.2.1.2, some of the samples were enzymatically treated to hydrolyze any conjugated metabolite. The samples were buffered to pH 7.0 with phosphate buffer and incubated at 37°C for 36 hours after the addition of β-glucuronidase (15 000 U/ml). Hydrolysis was terminated by the addition of 0.2 ml of 3 N perchloric acid.

4.4.2.3 Urinary Metabolites (*In Vivo* Hepatic Metabolism)

Forty-eight hour urine samples were collected from male, Sprague-Dawley rats (150 ± 20 g) administered with amphetamine (10 mg/kg, *ip.*). Prior to analysis for *p*-hydroxyamphetamine using assay Procedure B (Section 4.4.2.1.2), urine samples were treated in the manner described in Section 4.4.1.2.1, which involved; i) no pretreatment, ii) enzymic hydrolysis with β-glucuronidase, and iii) acid hydrolysis.

4.5 COMPANY LISTINGS

Abbott - Abbott Laboratories, Ltd., Montreal, Que.

Aldrich - Aldrich Chemical Company, Inc., Milwaukee, Wis.

B&L - Bausch & Lomb, Rochester, N.Y.

Bioscience Animal Services - Bioscience Animal Services, University of Alberta, Edmonton, Alberta.

Can Cab. - Canadian Cabinet Company, Ltd., Nepean, Ont.

Carl Zeiss - Carl Zeiss, Oberkochen/Wuertt., West Germany.

Clay Adams - Clay Adams, Parsippany, N.J.

Connaught - Connaught Laboratories, Ltd., Willowdale, Ont.

Corning - Corning Glass Works, Corning, N.Y.

Eastman - Eastman Kodak Company, Eastman Organic Chemicals, Rochester, N.Y.

EM - E. Merck Laboratories, Inc., Elmsford, N.Y.

FMC - FMC Corporation, Marine Colloids Division, Rockland, Md.

GIBCO - Grand Island Biological Company, Grand Island, N.Y.

Hewlett-Packard - Hewlett-Packard Instruments, San Diego, Ca.

KL - Koch-Light Laboratories Ltd., Colnbrook, England.

Leitz Wetzlar - E. Leitz (Canada) Ltd., Midland, Ont.

MCB - Matheson, Coleman, & Bell Manufacturing Chemists, Norwood, Ohio.

MP Corp. - Millipore Corporation, Freehold, N.J.

Nunc - Nunc, Roskilde, Denmark.

Organon - Organon Canada Ltd., Toronto, Ont.

Olympus - Olympus Optical Company, Ltd., Tokyo, Japan.

P&B - Pfaltz & Bauer, Inc., Stamford, Conn.

Pierce - Pierce Chemical Company, Rockford, Ill.

Polaroid - Polaroid Corporation, Cambridge, Md.

SGE - Scientific Glass Engineering Pty, Ltd., Austin, Tex.

Sigma - Sigma Chemical Co., St. Louis, Mo.

SKF - Smith, Kline, and French Laboratories, Philadelphia, Penn.

Supelco - Supelco, Inc., Bellefonte, Penn.

Synth. DBP - Synthesized by D.B. Prelusky

Synth. FMP - Synthesized by Dr. F.M. Pasutto

Unicam - Pye Unicam Ltd., Cambridge, England.

Upjohn - Upjohn Company of Canada, Don Mills, Ont.

Varian - Varian Associates, Inc., Palo Alto, Ca.

Vandermeer Farms - A. Vandermeer Farms, Sherwood Park, Alta.

Woodland Lab. - Woodland Laboratories, Geulph, Ont.

5. RESULTS AND DISCUSSION

5.1 METABOLIC REDUCTION OF KETONE CONTAINING COMPOUNDS BY MAMMALIAN TISSUE

There has been some disagreement regarding many of the existing principles which have been associated with the metabolic reduction of compounds possessing ketone groups. This has no doubt resulted from generalizations being formed on the basis of a limited number of observations. Conclusions based on these observations often lacked adequate support. It appears however, that a major reason for the inconclusive results was not the inability to obtain suitably purified enzymes as was commonly cited, but rather a failure to properly comprehend the metabolizing systems which were available. The majority of investigations have been performed *in vitro* utilizing heterogenous enzyme preparations. Thus, because of the artificial nature of these systems, it would be unrealistic to assume that results were dependent solely on the presence or absence of the necessary enzymes. Yet few investigators, when forming their conclusions, took into account how various experimental conditions could have affected the observed enzyme activities.

The current investigation was undertaken to examine the mechanisms involved during the metabolic reduction of ketones and many of the factors which influence this process. In doing so, it was possible to evaluate several of the general criteria which have been routinely accepted in in vitro drug metabolism studies.

5.1.1 In Vitro Metabolism of Propiophenone and Phenylacetone

A comprehensive examination of the in vitro mammalian reduction of two representative ketone-containing compounds was carried out. Propiophenone (I), an aromatic ketone, and its non-aromatic homolog phenylacetone (III), were used as the model substrates. The initial investigation involved a preliminary analysis of the extent to which these two ketones (I, III) were reduced to their corresponding alcohols (1-phenyl-1-propanol, II; 1-phenyl-2-propanol, IV, respectively) under standard incubation conditions. The occurrence of other metabolic reactions was also investigated. For comparison, 10 000Xg liver supernatants from two species were utilized as the enzyme sources: the rabbit, which has been shown to be very efficient in catalyzing the reduction of ketonic compounds, and the rat, which shows relatively weak reductase activity (10). Metabolic products formed by reduction, hydroxylation, or alternative mechanisms were identified and quantitated.

5.1.1.1 Identification of Metabolites

Identification of each metabolic product was confirmed primarily by direct comparison of gas chromatographic and mass spectral characteristics with those of an authentic standard. Tentative assignment of chemical structures based on fragmentation profiles was required prior to acquisition of the authentic standards. Compounds established as *in vitro* metabolites of either propiophenone (7) or phenylacetone (III) are given in Table 7. The metabolic profiles of both substrates are also illustrated (Fig. 19).

5.1.1.1.1 Gas Liquid Chromatography

GLC analyses of the concentrated extracts from the *in vitro* incubation mixtures were carried out according to the operating parameters described in Table 6. Due to the varied chromatographic properties of the recovered metabolites, it was necessary to employ three GLC systems to ensure that a comprehensive search was performed. For some analyses, an OV-101 column packing material was used (System A). This is a non-polar silicone-type stationary phase, on which separation of chemically related compounds is essentially on the basis of their volatility. Thus, a series of alcohols will separate with the lowest boiling member eluting first, and a series of ketones will behave similarly. It is however, generally not possible to predict the degree of resolution of mixtures of alcohols and ketones. Using System A, it proved possible to separate the ketols (VI and VII) from the diol (VIII), but it was not possible to resolve adequately either ketone (I or III) from its corresponding alcohol (II or IV). A typical gas-liquid chromatogram is illustrated in Figure 20. When a more polar column packing

TABLE 7. Gas Liquid Chromatographic (GLC) Retention Times for Propiophenone (I), Phenylacetone (III), and Their Metabolites.

SUBSTRATE		**RETENTION TIME (min.)**			
	column system:[1]	A		B	C
	temperature:[2]	95°	120°	120°	90°
(IS)[3]	CYCLOHEXYLACETONE	3.50	1.05	1.05	—
(IS)	ALLYLBENZENE	—	—	—	2.05
III	PHENYLACETONE (A)	3.85	1.25	4.05	12.90
IV	1-PHENYL-2-PROPANOL (B)	3.90	1.30	6.15	6.05*
I	PROPIOPHENONE (C)	4.70	1.50	3.85	14.50
II	1-PHENYL-1-PROPANOL (D)	4.80	1.50	7.75	4.95*
V	1-PHENYL-1,2-PROPANEDIONE (H)	5.40	1.60	5.85	15.90
VI	1-HYDROXY-1-PHENYL-2-PROPANONE (E)	9.20	2.10	23.10	17.75*
VII	2-HYDROXY-1-PHENYL-1-PROPANONE (F)	11.35	2.40	20.35	27.30*
VIII	1-PHENYL-1,2-PROPANEDIOL (G)[4]	21.75^{T} 24.10^{E}	3.35	▬[5] ▬	9.95 *^{T} 8.10 *^{E}
IX	BENZALDEHYDE	1.10	.50	1.75	17.95
X	BENZYL ALCOHOL	2.50	.90	8.65	2.80*
XI	BENZOIC ACID	▬	33.00	▬	▬

---

[1] FOR EXPLANATION OF GLC COLUMN SYSTEMS SEE TABLE 6.

[2] OVEN TEMPERATURE.

[3] IS - INTERNAL STANDARD.

[4] *T* = *threo* DIASTEREOISOMER; *E* = *erythro* DIASTEREOISOMER.

[5] ▬ = COMPOUND NOT ELUTED ON THIS GLC SYSTEM.

FIGURE 19. *In vitro* metabolism of propiophenone (I) and phenylacetone (III), and suggested mechanisms of pathways. Metabolic ⟶ ; Chemical ----→

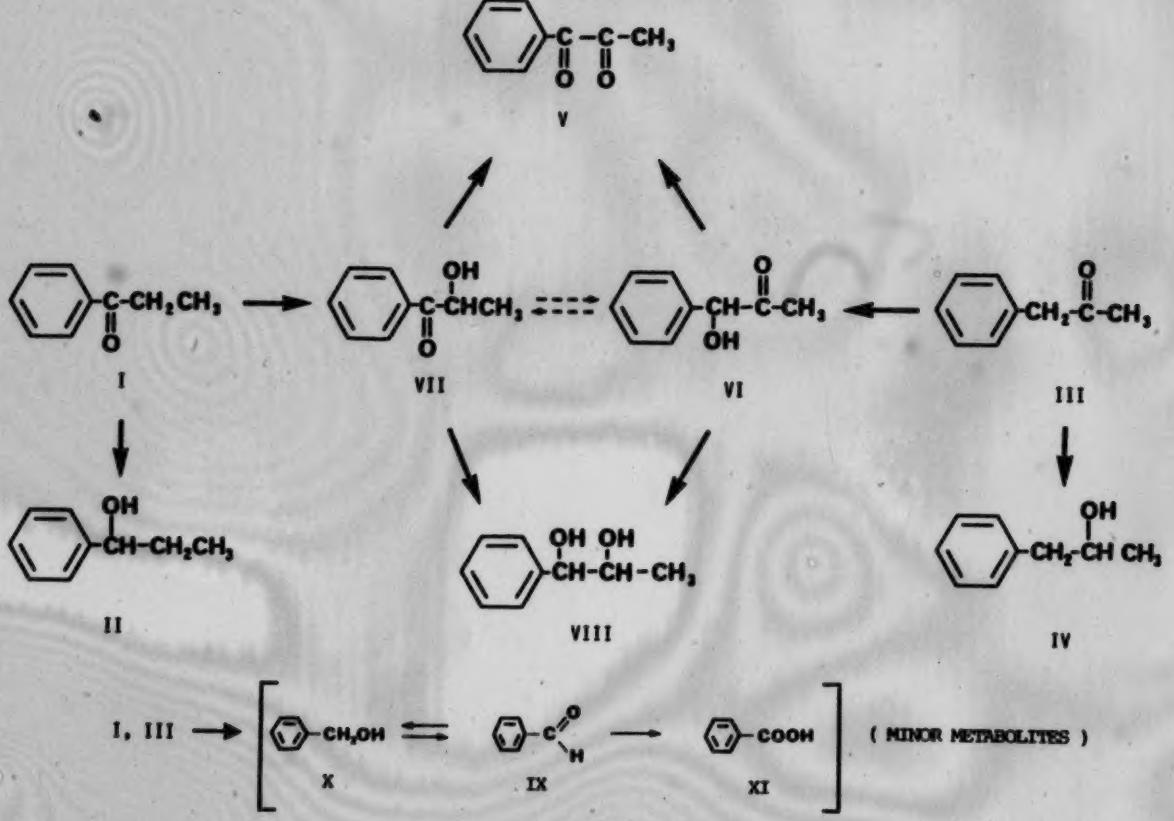
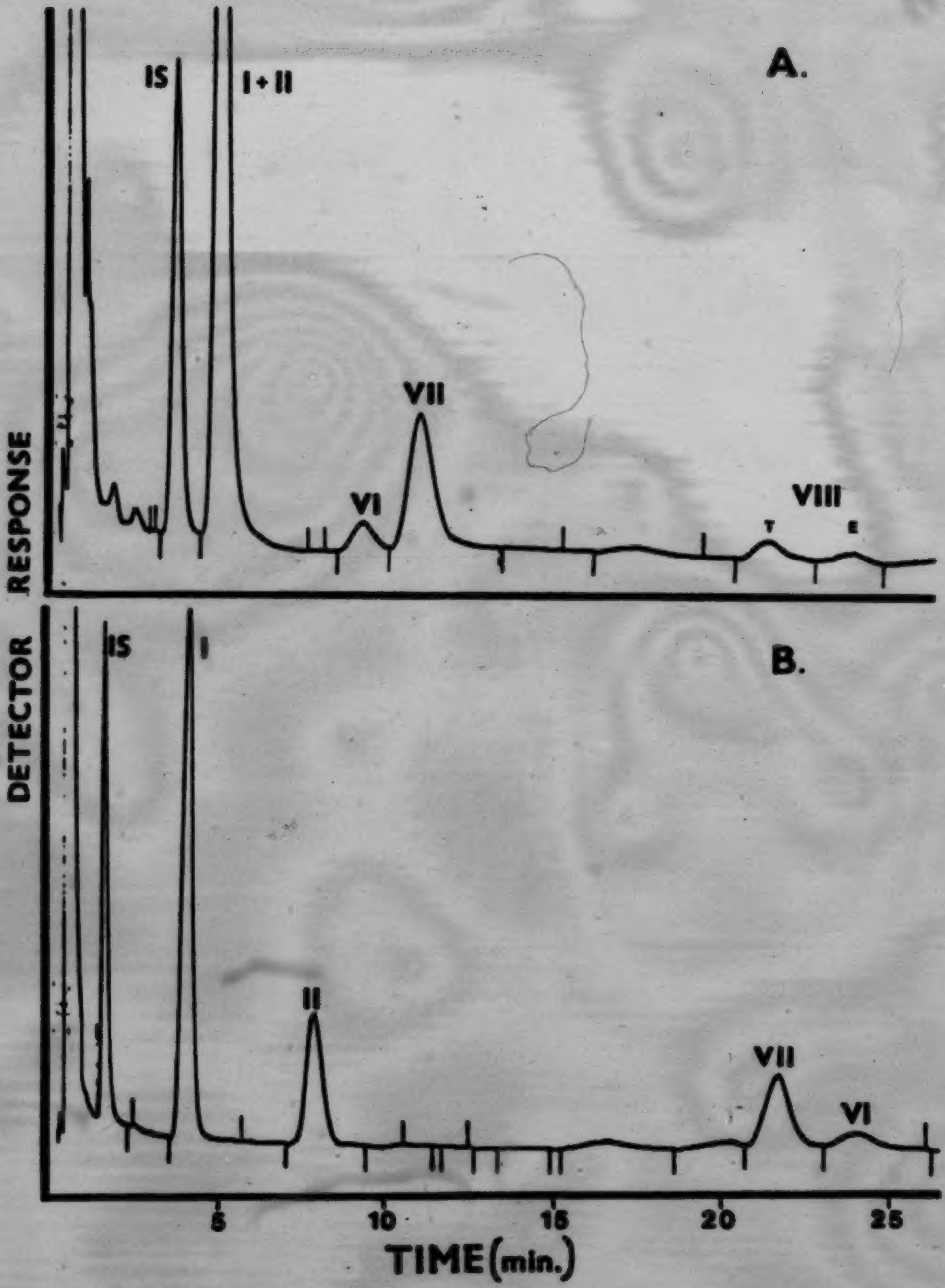

I
II
III
IV
V
VI
VII
VIII
I, III
X
IX
XI
(MINOR METABOLITES)

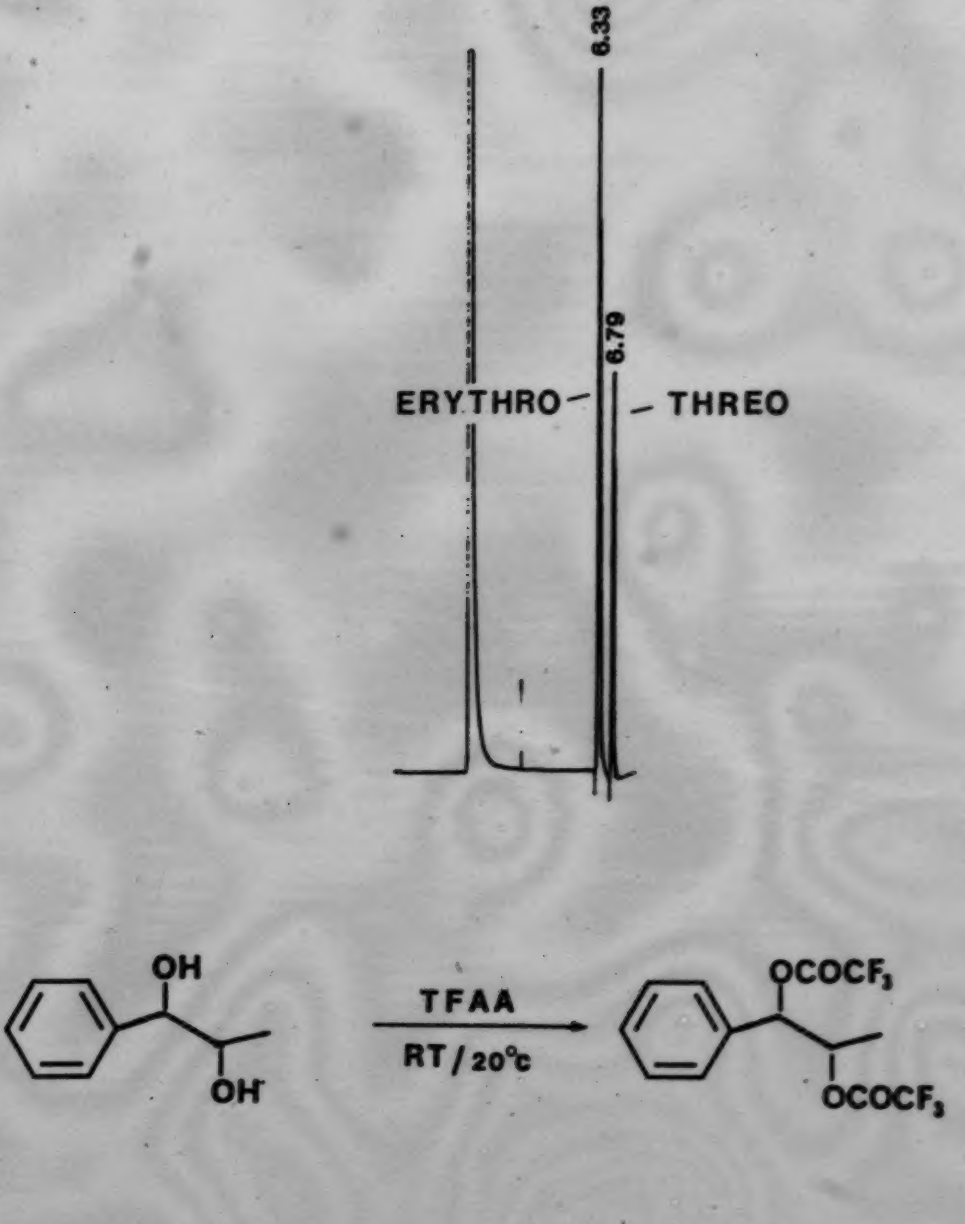
FIGURE 20. Gas liquid chromatogram of an ether/methylene chloride extract obtained from the *in vitro* incubation of propiophenone (I) in the presence of fortified rabbit liver 10 000X*g* supernatant: A) GLC System A, 5% OV-101; B) GLC System B, 2.5% Carbowax 20M, 2.5% KOH.



(Carbowax 20M, alkaline) was used (System B), these compounds (I and II, or III and IV) were completely resolved. A representative GLC trace is provided in Figure 20.

Since the erythro- and threo-diastereoisomers of 1-phenyl-1,2-propanediol (VIII) could only be partially resolved when the non-polar System A was used, attempts were made to find a more polar column to increase resolution. Several such liquid phases were tested, including XE-60, OV-225, OV-17, OV-3, and carbowax 20M. However, because of the extremely polar nature of these stationary phases, they could not be adequately separated on any of these stationary phases within a reasonable time period. Severe peak tailing resulting in poor peak integration was observed. In order to facilitate the analysis, the diastereoisomers (VIII) were converted to their O,O-di(trifluoroacetyl) derivatives by reacting the extract residue with TFAA prior to analysis by GLC. The resulting perfluoroacylated compounds exhibited decreased polarity and sufficient volatility to permit efficient resolution and considerably shorter retention times. For separation purposes, a moderately polar liquid phase (System C; OV-17) was selected. On this column, the TFA-derivatives had excellent chromatographic properties (Fig. 21), and remained stable even at high GLC operating temperatures.

FIGURE 21. Gas liquid chromatographic separation of the *erythro* and *threo* diastereoisomers of 1-phenyl-1,2-propanediol (VIII) following derivatization with trifluoroacetic anhydride. (TFAA).

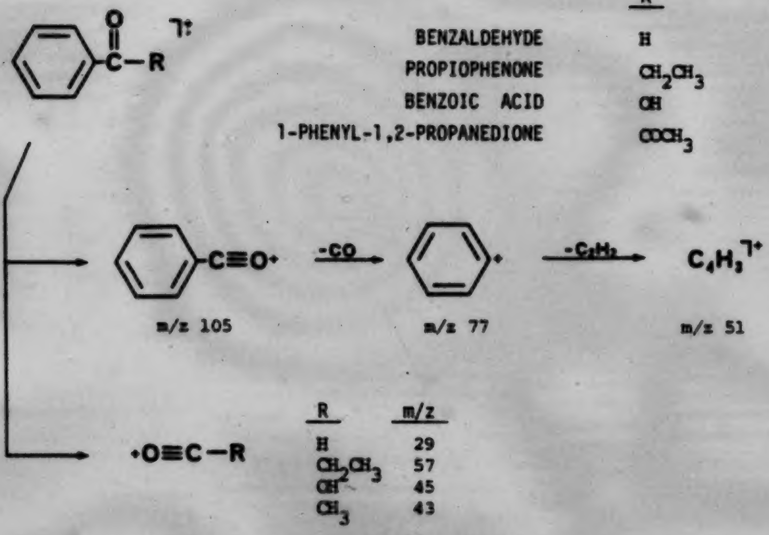


5.1.1.1.2 Mass Spectrometry

Electron impact mass spectrometry (EI-MS) was used for the characterization of products formed during drug metabolism studies. In most instances, interpretation of mass spectral data was uncomplicated. Structural identities were derived from first principle fragmentation reactions of electron impact ionized compounds. Diagnostic ions and plausible fragmentation pathways of metabolites; propiophenone (I), 1-phenyl-1,2-propanedione (V), benzaldehyde (IX), and benzoic acid (XI) (Fig. 22), 1-phenyl-1-propanol (II) and benzyl alcohol (x) (Fig. 23); phenylacetone (III) and 1-phenyl-2-propanol (IV) (Fig. 24) are given.

Tentative identification of metabolite E (Table 7), a metabolite of phenylacetone, was made on the basis of its mass spectral behavior, which was similar to that of other compounds examined. Diagnostic peaks in the spectrum were those of m/z 107, 105, 79 and 77 (Fig. 25) which suggested that compound E was a derivative of benzyl alcohol (Ph-CHOH-R) (cf Fig. 23). The mass spectrum also contained an additional characteristic fragment of m/z 43 ($CH_3{\equiv}O^+$) and a weak molecular ion at m/z 150 ($M^{+\cdot}$). These ions were compatible with R in Figure 22 being a methyl ketone group, therefore identifying metabolite E as 1-hydroxy-1-phenyl-2— propanone (VI). An authentic sample of (±)-VI was synthesized (Section 4.1.1.3.1) and had GLC/MS properties identical to those of metabolite E.

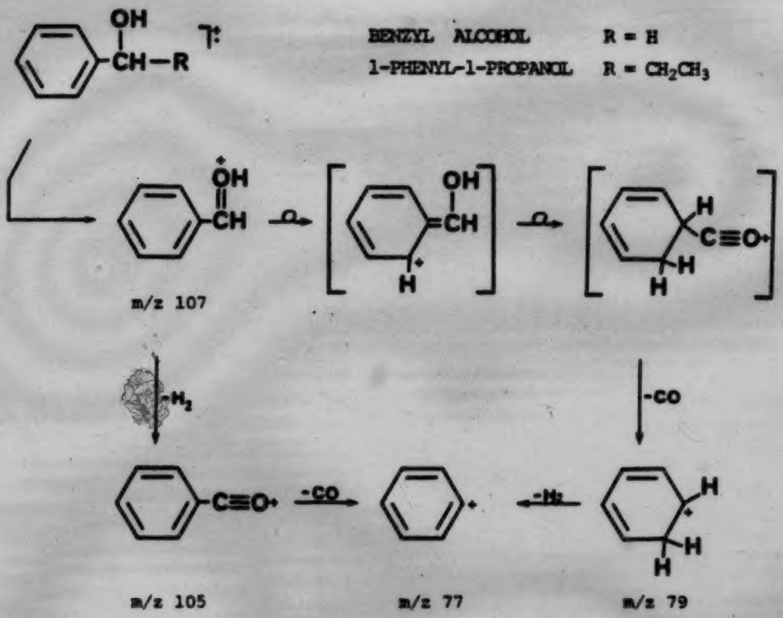
FIGURE 22. EI-MS fragmentation profiles of propiophenone (I), 1-phenyl-1,2-propanedione (V), benzaldehyde (IX), and benzoic acid (XI).


O
C—R
R
BENZALDEHYDE H
PROPIOPHENONE CH_2CH_3
BENZOIC ACID OH
1-PHENYL-1,2-PROPANEDIONE $COCH_3$
C≡O·
-CO
·
$-C_2H_2$
C_4H_3
m/z 105
m/z 77
m/z 51
·O≡C—R
R m/z
H 29
CH_2CH_3 57
OH 45
CH_3 43

% RELATIVE ABUNDANCE

m/z	R= H	R= CH_2CH_3	R= OH	R= $COCH_3$
148	--	--	--	-- (M†)
134	--	23 (M†)	--	--
122	--	--	72 (M†)	--
106	93 (M†)	--	--	8
105	100	100	100	100
77	43	43	54	75
57	--	1	--	--
51	3	5	10	13
45	--	--	19	--
43	--	--	--	9
29	2	--	--	--

FIGURE 23. EI-MS fragmentation profiles of 1-phenyl-1-propanol (II) and benzyl alcohol (X).

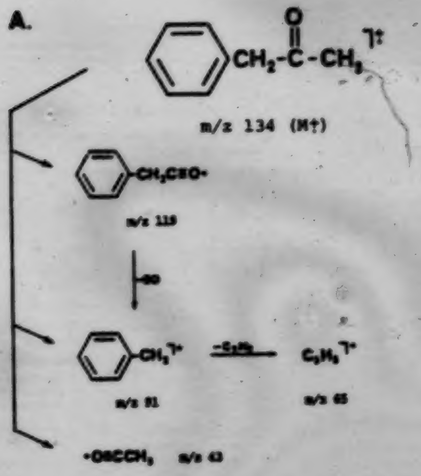

BENZYL ALCOHOL R = H
1-PHENYL-1-PROPANOL R = CH2CH3
m/z 107
-H2
-CO
m/z 105
-CO
m/z 77
-H2
m/z 79

% RELATIVE ABUNDANCE

m/z	R = H	R = CH_2CH_3
136	-	19 ($M^{\dot{+}}$)
108	99 ($M^{\dot{+}}$)	-
107	60 $(M-1)^+$	100
105	17	8
79	100	79
77	46	35

FIGURE 24. EI-MS fragmentation profiles of: A) phenylacetone (III); B) 1-phenyl-2-propanol (IV).

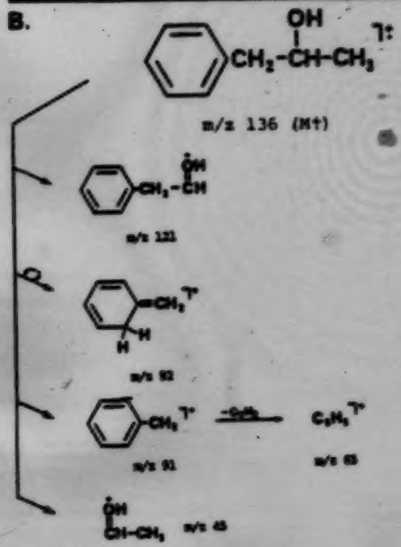
A.


m/z 134 (M+)
m/z 119
-CO
m/z 91
C5H5
m/z 65
·O≡CCH3 m/z 43

PHENYLACETONE

m/z	% Rel. Ab.
134 (M+)	47
119	4
91	100
65	26
43	33

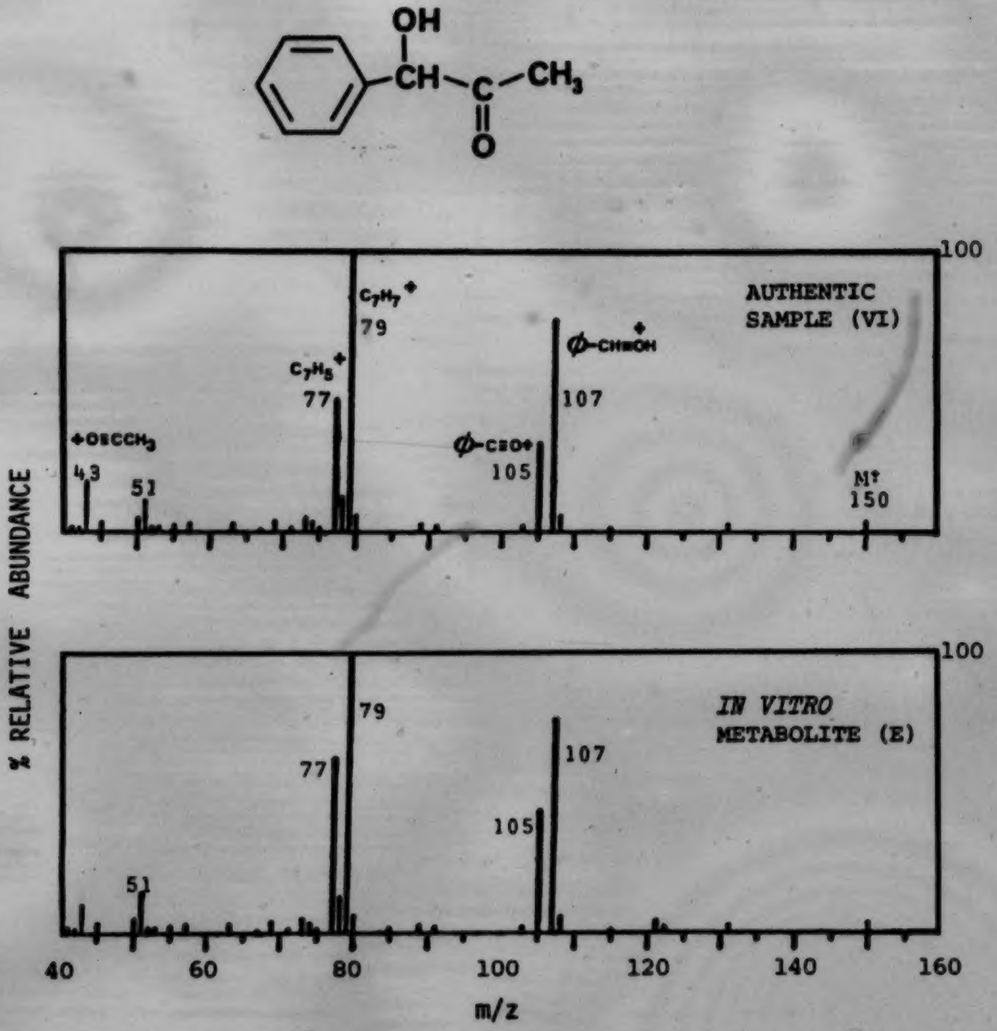
B.


m/z 136 (M+)
m/z 121
m/z 92
m/z 91
C5H5
m/z 65
m/z 45

1-PHENYL-2-PROPANOL

m/z	% Rel. Ab.
136 (M+)	3
121	3
92	100
91	66
65	15
45	4

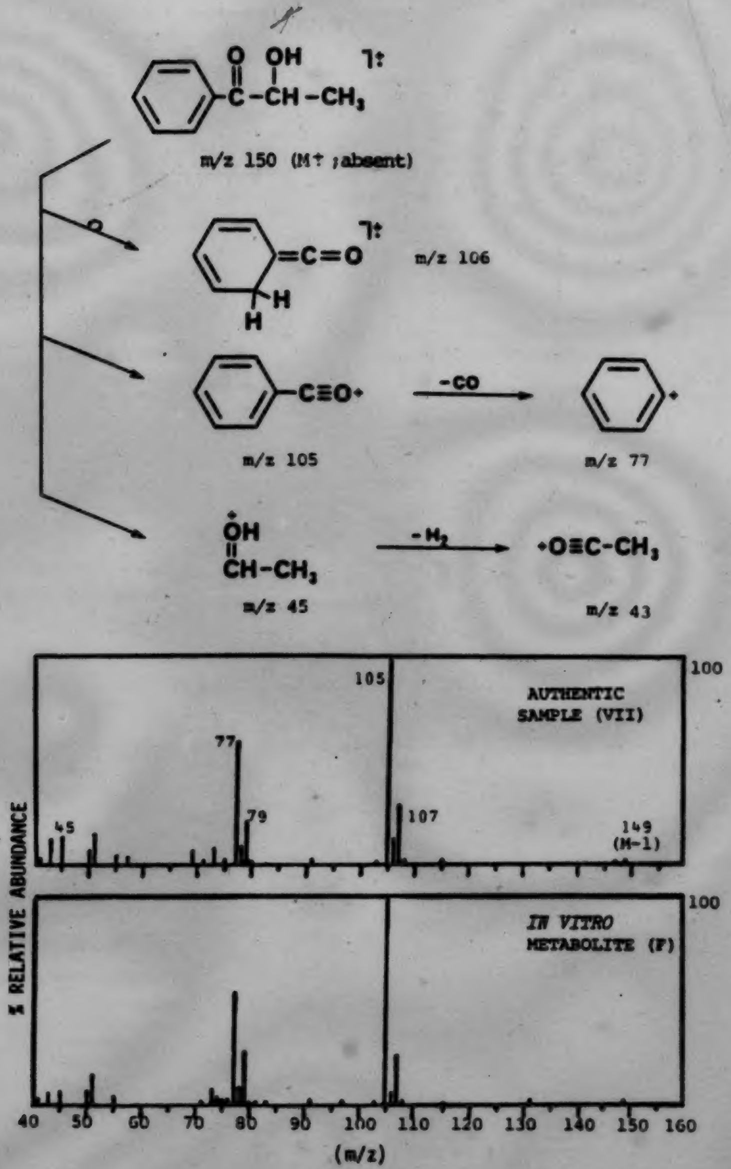
FIGURE 25. EI-MS identification of 1-hydroxy-1-phenyl-2-propanone (VI) as an *in vitro* metabolite (E) of phenylacetone (III).



Identification of the structure of metabolite F could not be deduced directly from its mass spectral fragmentation pattern (Fig. 26), which contained the same characteristic complex of ions (m/2 107, 105, 79, 77) that were observed in the spectrum of E (1-hydroxy-1-phenyl-2-propanone, Fig. 25), and also in the spectra of 1-phenyl-1-propanol and benzyl alcohol (Fig. 23). This similarly indicated that compound F contained a benzylic alcohol group. However, the relatively low abundances of the peaks at m/z 107 (28%) and m/z 79 (25%) were not consistent with the normal fragmentation behavior expected for benzylic alcohols. The presence of more prominent fragments at m/z 105 (base peak) and m/z 77 (61%) were to a greater extent typical of arylalkylketones (cf Fig. 22). This strongly indicated the presence of a carbonyl moiety adjacent to the aromatic ring rather than an alcohol group as the mass spectrum implied initially. A plausible assumption from this information was that metabolite F was 2-hydroxy-1-phenyl-1-propanone (VII), a structural isomer of compound E (1-hydroxy-1-phenyl-2-propanone, VI). Verification of this conclusion was obtained by direct comparison of the GLC/MS characteristics of F with an authentic synthetic sample of the suspected compound, VII (Section 4.1.1.3.2).

The presence of the ions of m/z 107 and 79 in the spectrum of metabolite F required explanation. It was concluded that their presence was due to the partial isomerization of

FIGURE 26. EI-MS identification of 2-hydroxy-1-phenyl-1-propanone (VII) as an *in vitro* metabolite (F) of propiophenone (I) and phenylacetone (III).



VII to 1-hydroxyl-1-phenyl-2-propanone (VI) during the GLC/MS process. This resulted in a composite mass spectrum of the two compounds and accounted for the unexpected fragments. This conclusion was further substantiated by several other observations. The chemical interconversion of these two ketols (VII $\rightleftharpoons$ VI) has been detected by others (447-449) and in the current study (Sections 4.1.1.3.1 and 4.1.1.3.2) during their chemical synthesis. In addition, solutions of these and related ketols have been observed to undergo isomerization on standing (448, 450-454). Therefore, it would be reasonable to assume that metabolically formed ketols would also interconvert. This was confirmed by the detection of 1-hydroxy-1-phenyl-2-propanone (VI) as an unexpected metabolite of propiophenone (Table 8), which in all probability resulted directly from the chemical conversion of 2-hydroxy-1-phenyl-1-propanone (VII) (Fig. 19). In further agreement with these studies, Speier (113) reported that α-hydroxy ketones probably exist in two tautomeric forms, which allowed easy interconversion of the two isomers through an enediol intermediate (Fig. 27).

Metabolite G (Table 7) was detected as an *in vitro* metabolite of both propiophenone (I) and phenylacetone (III). GLC/mass spectral analysis identified the compound as a mixture of the *erythro*- and *threo*-diastereoisomers of 1-phenyl-1,2-propanediol (VIII). The mass spectrum and

TABLE 8. *In Vitro* Metabolism of Phenylacetone (III) and Propiophenone (I) by NADPH-Fortified Rat and Rabbit Liver 10 000X*g* Supernatant.

		METABOLITE [c]						
		[b]		DIOL		KETOL		
SUBSTRATE	SPECIES	KETONE	ALCOHOL	*ERYTHRO*	*THREO*	VII	VI	DIKETONE [d]
PHENYLACETONE (III)	RAT	84.7 ± 2.5	3.2 ± 0.26	0.39 ± 0.18	0.90 ± 0.27	ND.[e]	1.06 ± 0.20	1.07 ± 0.30
	RABBIT	2.7 ± 0.24	93.7 ± 2.8	ND.	ND.	ND.	0.43 ± 0.14	TR[f]
PROPIOPHENONE (I)	RAT	63.1 ± 2.23	19.1 ± 1.1	0.23 ± 0.09	1.45 ± 0.28	13.1 ± 0.93	2.37 ± 0.43	0.60 ± 0.13
	RABBIT	5.5 ± 0.61	75.3 ± 2.1	1.30 ± 0.22	2.05 ± 0.26	13.3 ± 1.43	0.94 ± 0.13	ND.

[a] INCUBATED WITH FORTIFIED LIVER 10 000Xg SUPERNATANT; FORTIFIED WITH AN NAPDH-GENERATING SYSTEM (NADP+, GLUCOSE-6-PHOSPHATE, $MgCl_2$): THREE EXPERIMENTS RUN IN TRIPLICATE OR QUADRUPLICATE FOR EACH SET OF CONDITIONS.

[b] KETONE AND CORRESPONDING ALCOHOL; PHENYLACETONE (III) AND 1-PHENYL-2-PROPANOL (IV),
PROPIOPHENONE (I) AND 1-PHENYL-1-PROPANOL (II).

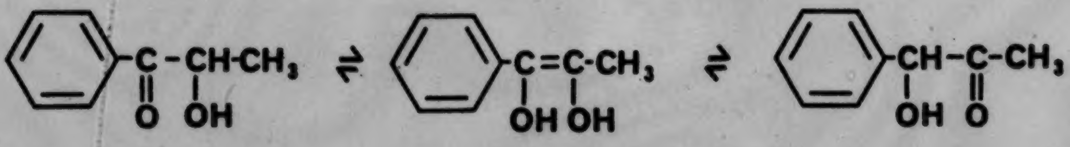
[c] PERCENTAGE RECOVERY (MEAN ± STANDARD DEVIATION; n = 9 or 12)

[d] INCUBATED WITH RESUSPENDED 105 000Xg MICROSOMAL PELLET

[e] NOT DETECTED.

[f] TRACE < 0.2%

FIGURE 27. Interconversion (tautomerism) of 1-hydroxy-1-phenyl-2-propanone (VI) and 2-hydroxy-1-phenyl-1-propanone (VII) through an enediol intermediate.



VII 'ENEDIOL' VI

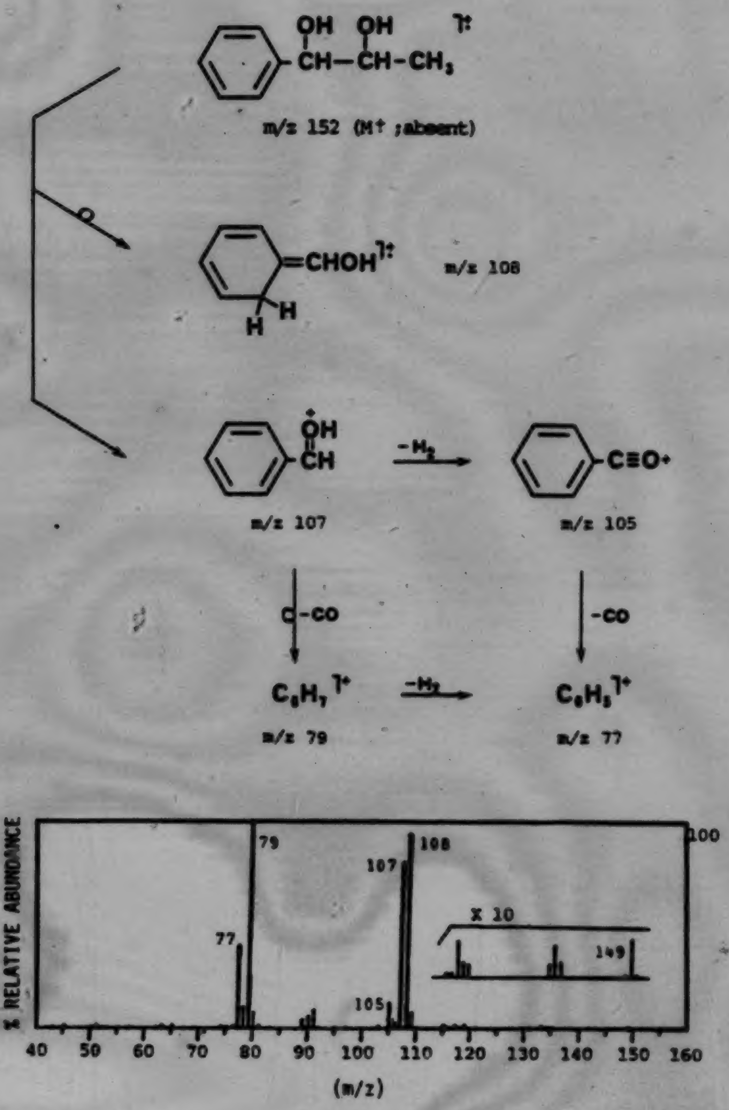
plausible fragmentation pattern for VIII are shown in Figure 28. The mass spectra of the two isomers of VIII (erythro, threo) were identical.

5.1.1.2 Quantitation of Metabolites and Determination of Metabolic Pathways

5.1.1.2.1 Metabolic Reduction

As demonstrated with both ketone substrates (I, III) (Table 8) the reductase activity found in rabbit liver preparations was in general, considerably greater than the activity of rat liver. The non-aromatic ketone phenylacetone (III) was reduced to its alcohol, 1-phenyl-2-propanol (IV), only to a minor extent (3%) when incubated for 60 minutes in the presence of 10 000X*g* rat liver supernatant fortified with an NADPH-generating system. Under the same conditions, when a rabbit liver preparation was used, 94% of the ketone (III) was converted to the alcohol (IV). Similarly, whereas reduction of the aromatic ketone, propiophenone (I), produced about 19% of its corresponding alcohol (1-phenyl-1-propanol II) when a rat liver 10 000X*g* supernatant preparation was used, in the presence of 10 000Xg rabbit liver supernatant greatly increased amounts (75%) of II were formed. Other investigators have reported similar results for the NADPH-catalyzed reduction of phenylacetone (III) by rabbit liver preparations (455-458).

FIGURE 28. EI-MS fragmentation profile of 1-phenyl-1,2-propanediol (VIII).


OH OH
CH—CH-CH₃
m/z 152 (M† ;absent)
a
=CHOH
m/z 108
H
H
ȮH
CH
m/z 107
-H₂
C≡O+
m/z 105
c -CO
-CO
C₆H₇
-H₂
C₆H₅
m/z 79
m/z 77
% RELATIVE ABUNDANCE
79
108
107
77
105
X 10
149
100
40
50
60
70
80
90
100
110
120
130
140
150
160
(m/z)

5.1.1.2.2 Metabolic C-Hydroxylation

In vitro incubations of either ketone substrate (I, III) were found to produce several other metabolites in addition to the corresponding alcohols (Table 8). The isolation of small amounts of 1-hydroxy-1-phenyl-2-propanone (VI) and 1-phenyl-1,2-propanediol (VIII) as products of phenylacetone (III) incubated with an NADPH-fortified rat liver preparation implied that hydroxylation at the benzylic methylene group had occurred (Fig. 19). A similar reaction was not observed in the presence of the 10 000X*g* rabbit liver preparation although trace amounts of VI were produced when the microsomal fraction (105 000X*g* resuspended pellet) was substituted for the liver supernatant. In contrast, propiophenone (I) was extensively metabolized to other hydroxylated products in addition to II, regardless of species (Table 8). The major oxidative metabolite isolated from either rat or rabbit liver incubations was 2-hydroxy-1-phenyl-1-propanone (VII). Minor amounts of 1-hydroxy-1-phenyl-2-propanone (VI) and of an isomeric mixture of threo- and erythro-1-phenyl-1,2-propanediol (VIII) were also recovered.

5.1.1.2.3 In Vitro Metabolic Fate of Propiophenone and Phenylacetone

The in vitro conversion of phenylacetone (III) to 1-phenyl-1,2-propanediol (VIII) has been proposed to occur by either hydroxylation of the alcohol (1-phenyl-2-propanol, IV) or through reduction of the ketol (1-hydroxy-1-phenyl-2-propanone, VI). Based on their observations, Kammerer *et al*. (455) favored the former route (III ⟶ IV ⟶ VIII). However, results obtained in the present study did not support their conclusion. Incubation of the alcohol (IV) in the presence of either rat or rabbit liver preparation (10 000X*g* supernatant) fortified with an NADPH-generating system failed to produce the diol (VIII), thus indicating that 1-phenyl-2— propanol (IV) was not a required precursor in the formation of 1-phenyl-1,2-propanediol. Additional evidence favors production of the diol by the alternative mechanism (III ⟶ VI ⟶ VIII) (Fig. 19). Metabolism of phenylacetone (III) by fortified liver microsomal preparations resulted in recovery of 1-hydroxy-1-phenyl-2-propanone (VI), indicating that this ketol (VI) was formed directly from III through microsomal *C*-hydroxylation. Further substantiation for this pathway is provided by the study of Beckett *et al*. (459) who observed that the ketol (VI) produced by the metabolic deamination of norephedrine by a fortified rabbit liver preparation underwent subsequent metabolic reduction to a diastereoisomeric mixture of 1-phenyl-1,2-propanediol (VIII). Related *in vivo* rabbit studies on norephedrine by Sinsheimer *et al*. (460) also showed substantial recoveries of the diol (VIII) and its precursor ketol compound (VI).

The in vitro metabolism of propiophenone (I) also resulted in low levels of 1-hydroxy-1-phenyl-2-propanone (VI) being detected (Table 8). This ketol (VI) is an isomer of the already identified metabolite 2-hydroxy-1-phenyl-1-propanone (VII), from which it was probably formed by chemical rearrangement (Section 5.2.1.2).

The diol (VIII) produced from propiophenone (I) was formed through reduction of the corresponding ketol (VII) rather than by direct hydroxylation of the alcohol, 1-phenyl-1-propanol (II) (Fig. 19). This conclusion was confirmed by demonstrating that II was not a precursor for metabolic formation of 1-phenyl-1,2-propanediol (VIII).

5.1.1.3 Optimization of In Vitro Incubation Conditions

Investigations carried out utilizing in vitro models have a definite advantage compared to the in vivo system in that there is a more rigid control over many of the parameters which affect enzymic activity. A review of past studies which have relied on tissue supernatants or subcellular fractions has revealed a wide variation in experimental procedures and incubation conditions. Unfortunately this makes direct comparison between reported data and current findings difficult to interpret, as experience has demonstrated that even minor changes in methodology may significantly alter in vitro activity. A major objective of any in vitro drug metabolism study, therefore, should be the design of a model which maintains conditions for optimal enzymic activity. The subsequent experiments pertained to determining those conditions.

Studies involved the two representative ketonic compounds, propiophenone (I) and phenylacetone (III). Modifications were made to the experimental system to determine their effect on the metabolic reduction and aliphatic hydroxylation of the substrates. An evaluation of the function of NADP and NAD in the incubation system was carried out. The effects of the presence (or absence) of glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), and nicotinamide were also determined. The actual modifications made to the incubation mixture are given in Table 9 and are now discussed.

5.1.1.3.1 Cofactor Requirements

The results summarized in Table 9 indicate that metabolic reduction of both propiophenone (I) and phenylacetone (III) did occur to a small extent when these substrates were incubated in the presence of rat liver 10 000X*g* supernatant without additional cofactors. This was not unexpected as low levels of endogenous NADPH (and NADH) have been detected in the supernatant fraction of whole liver homogenates (461). However, the overall activity of these reactions was greatly enhanced upon either supplementing the incubation mixture with an in vitro NADPH- or NADH-generating

TABLE 9. Variations in the Extent of the *In Vitro* Metabolism (percent conversion ± S.D)[a] of Propiophenone (I) and Phenylacetone (III) as a function of the Changes made in the Incubation Mixture Components.

INCUBATION MIXURE [b]	COFACTOR	PROPIOPHENONE		PHENYLACETONE		COFACTOR	PROPIOPHENONE		PHENYLACETONE	
		REDUCTION (II)	HYDROXYLATION (VII)	REDUCTION (III)	HYDROXYLATION (VI)		REDUCTION (II)	HYDROXYLATION (VII)	REDUCTION (III)	HYDROXYLATION (VI)
NO COFACTOR	----	1.50 ± 0.16	0.30 ± 0.09	0.55 ± 0.15	TR [c]	----	ND. [d]	ND.	ND.	ND.
COFACTOR ONLY	NADPH	26.5 ± 2.20	16.0 ± 1.40	3.80 ± 0.25	1.75 ± 0.21	NADH	48.4 ± 3.20	2.23 ± 0.25	51.0 ± 2.30	0.37 ± 0.13
	NADP+	2.50 ± 0.31	0.32 ± 0.10	0.58 ± 0.18	TR	NAD+	1.80 ± 0.21	0.38 ± 0.12	1.01 ± 0.19	TR
G-6-P, $MgCl_2$	NADP+	17.5 ± 1.62	11.7 ± 1.03	3.10 ± 0.30	1.02 ± 0.20	NAD+	20.6 ± 1.90	7.80 ± 0.67	20.3 ± 1.30	0.44 ± 0.18
G6P, $MgCl_2$, G6PDH (BAKER'S YEAST)	NADP+	24.8 ± 2.10	14.4 ± 1.60	3.90 ± 0.30	1.43 ± 0.22	NAD+	19.2 ± 1.60	8.00 ± 0.97	18.7 ± 1.32	0.38 ± 0.21
G6P, $MgCl_2$, G6PDH (L. mesenteroides)	NADP+	23.1 ± 2.33	12.0 ± 1.25	3.55 ± 0.40	1.81 ± 0.21	NAD+	53.7 ± 2.97	7.52 ± 0.73	59.0 ± 0.18	0.50 ± 0.23
G6P, $MgCl_2$, 30 μmol NICOTINAMIDE	NADP+	15.8 ± 1.11	8.04 ± 0.75	3.11 ± 0.37	1.02 ± 0.19	NAD+	19.6 ± 2.06	6.00 ± 0.73	18.5 ± 1.78	0.26 ± 0.13
ISOCITRATE, $MgCl_2$, $MnCl_2$	NADP+	15.3 ± 1.30	9.70 ± 1.01	3.00 ± 0.31	1.65 ± 0.20	NAD+	4.31 ± 0.31	1.23 ± 0.27	2.63 ± 0.23	TR
ISOCITRATE, $MgCl_2$, $MnCl_2$, ISCDH	NADP+	24.9 ± 2.40	15.3 ± 1.33	3.70 ± 0.28	1.84 ± 0.19	NAD+	4.81 ± 0.22	1.26 ± 0.19	2.81 ± 0.28	TR

[a] S.D. - STANDARD DEVIATION ($n > 12$).

[b] INCUBATION MIXTURE: RAT LIVER 10 000Xg SUPERNATANT (equivalent to 0.2g original tissue), 1.0 ml; Tris-KCl BUFFER, 4.9 ml; SUBSTRATE (0.2 mol), 0.1 ml EtOH. Additional cofactors added in 0.1 ml Buffer.

[c] TR < 0.2%.

[d] ND. - NOT DETECTED.

system, or by the direct addition of the reduced form of these cofactors. This result concurred with earlier observations that metabolic reduction of ketonic compounds (I, III) was absolutely dependent on the availability of these coenzymes (199). Furthermore, only in their reduced state could NADP and NAD function as direct donors of the necessary reducing equivalents (hydrogen, electrons) required during this enzyme-mediated reaction. This was demonstrated by the inability to increase the levels of product alcohols (II, IV) when an incomplete generating system ($NADP^+$ or NAD^+, no G6P, no $MgCl_2$) was utilized. Similarly, the availability of the reduced form of either coenzyme (NADPH/NADH) was also shown as an absolute requirement for the aliphatic C-hydroxylation of both propiophenone (I) and phenylacetone (III) (Table 9). Omitting NADPH/NADH or adding cofactors in the oxidized form ($NADP^+/NAD^+$) only, essentially eliminated hydroxylase activity.

5.1.1.3.2 Cofactor Specificity

The extent to which the *in vitro* biotransformation of propiophenone (I) and phenylacetone (III) occurred was significantly dependent on which cofactor, NADP or NAD, was made available during the incubation procedure. It has already been described that some reductases specifically require NADP to function, whereas others can function only with NAD, and still others are capable of showing activity in the presence of either coenzyme (Section 3.1.2). This was further exemplified during the course of the present study (Table 9). Reduction of the aliphatic ketone compound, phenylacetone (III), was considerably greater using NADH (51%) than NADPH (4%), whereas its aromatic isomer, propiophenone (I), was substantially reduced in the presence of either cofactor, although NADH (48%) was also preferred over NADPH (26%).

In contrast with the varied cofactor specificity demonstrated by ketone reductases, NADPH was the more efficient electron donor in microsomal C-hydroxylation reactions, regardless of substrate (Table 9). When NADH was substituted for NADPH, hydroxylation of phenylacetone and propiophenone was decreased by 79% and 86% respectively. This paralleled previous reports which showed that by replacing NADPH with NADH as the sole source of electrons, the extent of product formation was normally inhibited by approximately 90% (69, 70).

5.1.1.3.3 NADPH/NADH-Generating Systems

In order to maintain sufficient levels of cofactor in its active reduced form, an auxiliary enzymic reaction which catalyzes the reduction of $NADP^+$ or NAD^+ is routinely employed in the incubation mixture (Fig. 29). This usually consists of the oxidized form of the cofactor ($NADP^+$/NAD^+) together with glucose-6-phosphate and $MgCl_2$ in the presence of a catalyzing enzyme, glucose-6-phosphate

FIGURE 29. Schematic representation for the generation of NADPH/NADH by the glucose-6-phosphate dehydrogenase enzyme system.

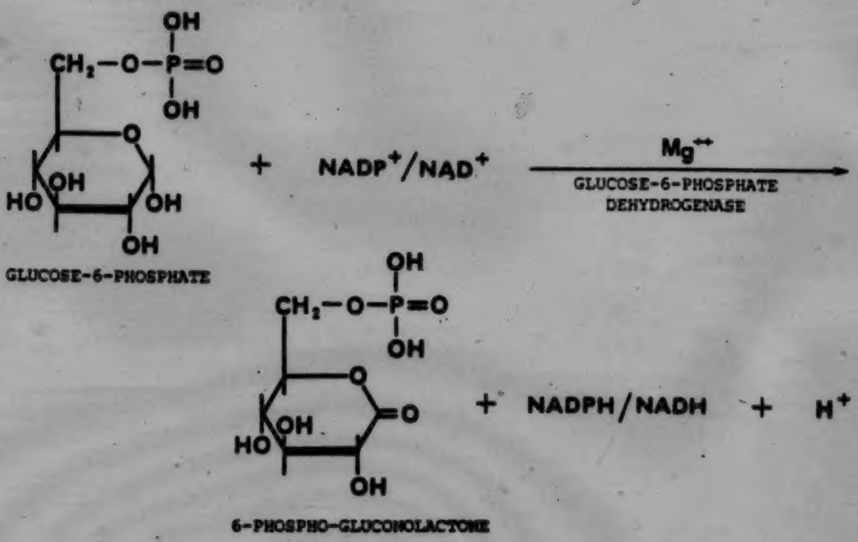
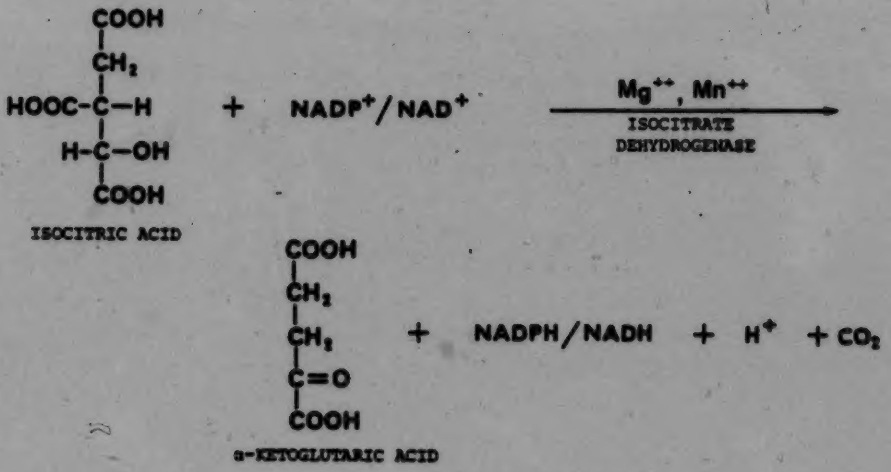

GLUCOSE-6-PHOSPHATE
NADP⁺/NAD⁺
Mg⁺⁺
GLUCOSE-6-PHOSPHATE DEHYDROGENASE
NADPH/NADH
H⁺
6-PHOSPHO-GLUCONOLACTONE

FIGURE 30. Schematic representation for the generation of NADPH/NADH by the isocitrate dehydrogenase enzyme system.


ISOCITRIC ACID
NADP⁺/NAD⁺
Mg⁺⁺, Mn⁺⁺
ISOCITRATE DEHYDROGENASE
NADPH/NADH
H⁺
CO₂
α-KETOGLUTARIC ACID

dehydrogenase (G6PDH). A commonly used alternative to the G6PDH system is the isocitric acid dehydrogenase system (ISCDH) (Fig. 30) (461). In the present study, it became apparent that reliance on an auxiliary coenzyme generating system did not always give optimal results.

5.1.1.3.3.1 Glucose-6-phosphate Dehydrogenase

The role of glucose-6-phosphate dehydrogenase (G6PDH) is to mediate the direct transfer of a hydride ion to $NADP^+$ /NAD^+ (Fig. 29) (462, 463). Several compounds are capable of serving as the source of this ion during the reaction, though D-glucose-6-phosphate (G6P) is the natural and most active substrate (462). For this reason G6P was used preferentially in experiments requiring generation of the reduced cofactors by a G6PDH catalyzed reaction.

In mammalian tissue, G6PDHs are cytoplasmic enzymes (462), consequently found in the 10 000X*g* supernatant and the soluble fraction (105 000X*g* supernatant) of liver (69, 464-470) and other sources such as brain, adrenal, mammary, and adipose tissue (471, 472). As a result, numerous studies have accepted the presence of the liver supernatant fraction in the incubation mixture as being an adequate source of G6PDH. However, in the current investigation, the absence of sufficient levels of endogenous G6PDH became apparent when the necessary amounts of NADPH for use in metabolic enzyme reactions could not be generated. This was illustrated when commercially reduced NADP (NADPH), used to fortify the incubation mixture, was replaced with an NADPH-generating system containing no additional G6PDH. The extent of metabolic reduction of propiophenone (I) and phenylacetone (III) was effectively decreased by 34% and 19%, respectively (Table 9). However, when 10 units of G6PDH (Baker's Yeast) were added along with the generating system, levels of metabolites were comparable to those detected when NADPH was directly utilized. A similar effect was observed with microsomal-mediated oxidations of both ketone substrates. In the absence of concurrent addition of commercial G6PDH (Baker's Yeast) to the NADPH-generating system, levels of *C*-hydroxylated metabolites from propiophenone were decreased 27%, and 42% when phenylacetone was the substrate (Table 9).

G6PDH, in addition to reducing NADP+, was also thought to be capable of efficiently reducing NAD+ (469, 473-475). However, Geisler *et al*. (476) demonstrated that while the endogenous G6PDH present in rat muscle, liver, or adipose tissue was able to catalyze the reduction of NADP+, NAD+ could only be efficiently reduced by the type of G6PDH found in adipose tissue. The G6PDH of rat liver exhibited only a very weak specificity towards NAD+. These findings were consistent with current results (Table 9) which revealed that the reduction of the ketonic substrates (I, III) in the presence of rat liver 10 000X*g* supernatant was approximately 2 1/2 times higher when NADH was added directly than when an NADH-generating system was used. This demonstrated a severe limitation in generating adequate levels of NADH when rat liver supernatant served as the sole source of G6PDH.

The requirement for a NAD-linked dehydrogenase could not be fulfilled by the addition of Baker's Yeast G6PDH as was possible with NADP+. The addition of 10 umol of exogenous G6PDH (Baker's Yeast) to the NADH-generating system caused little improvement in the extent to which reduction or *C*-hydroxylation occurred with either substrate (I, III). This effect was not totally unexpected. It is known that yeast G6PDH is an NADP-linked dehydrogenase which cannot utilize NAD+ (130). This problem though, was overcome by replacing Baker's Yeast G6PDH with a dual nucleotide specific dehydrogenase (NADP- and NAD-linked activity) isolated from a microbial source (*Leuconostoc mesenteroides*). Addition of this G6PDH to the NADH-generating incubation mixture increased metabolism (reduction and *C*-hydroxylation) of both substrates to an equal or greater extent than what was observed when NADH alone was added. Furthermore, *Leuconostoc mesenteroides* G6PDH also proved superior to Baker's Yeast G6PDH as the catalyst in the *in vitro* reduction of NADP+ (Table 9).

5.1.1.3.3.2 NADH-Synergism

It is known that NADH is an inadequate coenzyme for microsomal C-hydroxylation, being able to support a reaction rate only 10-15% of that observed with NADPH (69, 70, 81). This was further demonstrated in the current investigation when the reduced forms of NADPH and NADH were compared for their ability to mediate the enzymic hydroxylation of the substrates (I, III) (Table 9). In view of this observation, it was surprising to note that substitution of the NADPH-generating system with an NADH-generating system resulted in a considerably higher level of microsomal activity than the 10-15% expected (Table 9). The degree to which hydroxylation of propiophenone (I) occurred in the presence of an NADH-generating system was 67% of the level observed with the NADPH-generating system, 49% compared to when NADPH was added directly, and an incredible 350% of the level detected when NADH was added directly. The corresponding extent to which hydroxylation of phenylacetone (III) occurred with an NADH-generating system was 38%, 33%, and 157% of what was detected with the NADPH-generating system, the direct addition of NADPH, and the direct addition of NADH, respectively.

There was no obvious explanation of why substantial levels of hydroxylase activity occurred with the NADH-generating system and not when NADH was added directly to the incubates. It was therefore, considered informative to establish some idea concerning the composition of 10 000X*g* liver supernatant (other than metabolizing enzymes) which may have contributed to these unexpected results.

As incubations of propiophenone (I) with unfortified 10 000X*g* liver supernatant did show a low level of metabolic activity (Table 9), this was assumed to be due to the availability of low amounts of endogenous NADPH for utilization by metabolizing enzymes, if only to a very limited extent. When propiophenone (I) was incubated with the same 10 000X*g* liver preparation, but supplemented with glucose-6-phosphate (G6P) and $MgCl_2$ (*no* NADP$^+$), a comparatively higher rate of metabolism was detected then with tissue alone (Table 10). This increase was interpreted as the direct result of making more NADPH available when G6P (and $MgCl_2$) was present in the incubation mixture than when omitted. When dealing with sub-optimal concentrations of NADPH/NADH, the rate of drug metablized is directly proportional to the amount of cofactor available (461). If it was assumed that endogenous NADP/NAD actually exists predominantly in the oxidized state (NADP$^+$/NAD$^+$) and requires transformation to the active form (NADPH/NADH) prior to its utilization, a limiting step in the production of endogenous NADPH would presumably be the availability of G6P necessary to complete the transformation.

Thus, one explanation for the significant level of metabolic hydroxylation in the presence of the NADH-generating system

TABLE 10. Variations in the Extent of the *In Vitro* Reduction and Hydroxylation of Propiophenone, as a function of Changes made in the Incubation Mixture Components. The Source of Cofactors used (NADP/NAD) were Endogenous.

INCUBATION MIXTURE [a]	PERCENT METABOLISM ± S.D.[b] PROPIOPHENONE REDUCTION (II)	PERCENT METABOLISM ± S.D.[b] PROPIOPHENONE HYDROXYLATION (VII)
10 000Xg SUPERNATANT ONLY (BLANK)	1.20 ± 0.16	0.30 ± 0.09
$MgCl_2$	1.36 ± 0.11	0.33 ± 0.07
G6P	2.51 ± 0.55	0.85 ± 0.18
G6P, $MgCl_2$	2.83 ± 0.41	0.81 ± 0.21
G6PDH: (L. mesenteroides)	4.50 ± 0.51	2.17 ± 0.20
G6P, $MgCl_2$, G6PDH: (L. mesenteroides)	12.6 ± 1.30	6.31 ± 0.43
G6PDH: (BAKER'S YEAST)	3.82 ± 0.45	1.55 ± 0.22
G6P, $MgCl_2$, G6PDH: (BAKER'S YEAST)	10.9 ± 1.05	4.30 ± 0.58

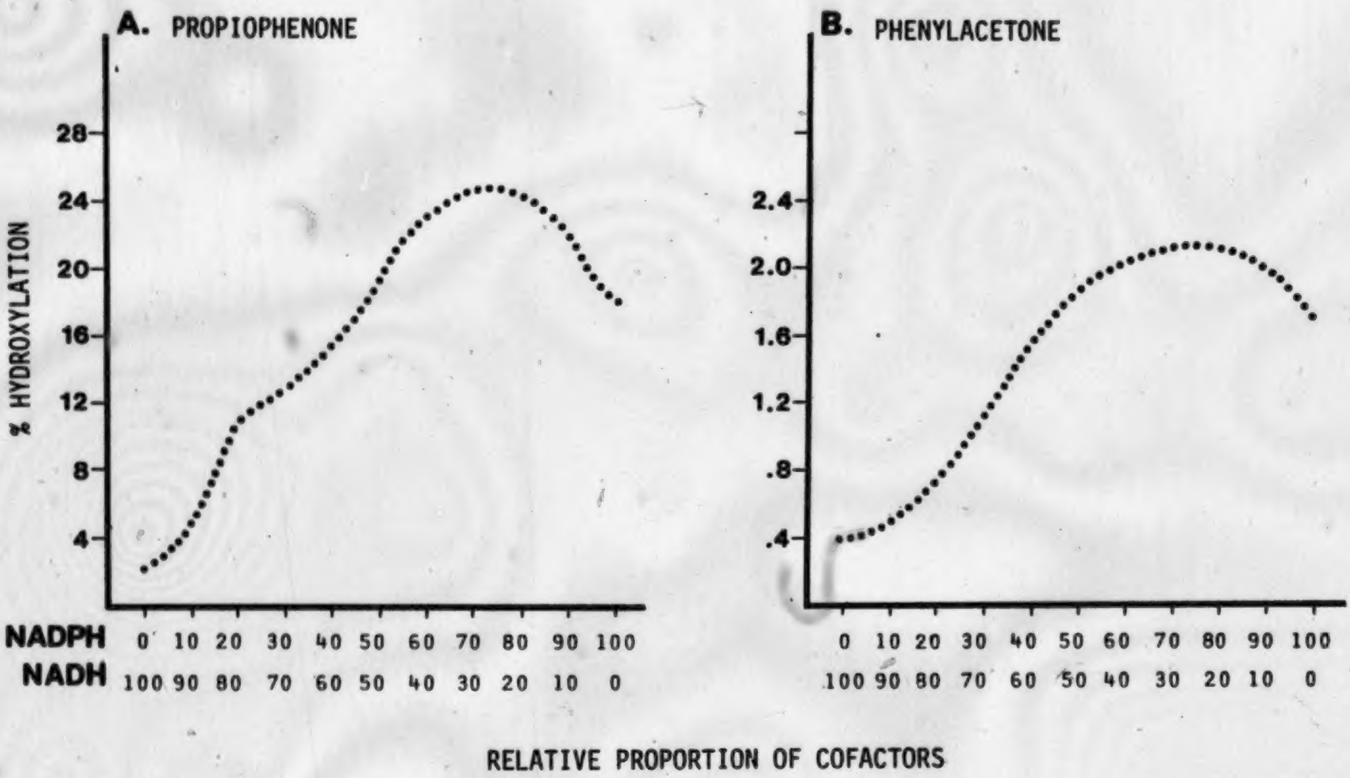
[a] BLANK INCUBATION MIXTURE CONSISTS OF: TRIS-KCl BUFFER (4.9 ml); RAT LIVER 10 000Xg SUPERNATANT EQUIVALENT TO 0.2 g ORIGINAL TISSUE (1 ml); SUBSTRATE 0.2 μmol (0.1 ml). ADDITIONAL COMPONENTS ADDED IN 0.1 ml BUFFER.

[b] S.D. = STANDARD DEVIATION; n = 12.

(NADP+, G6P, $MgCl_2$), was that G6P (and $MgCl_2$) also induced reduction of endogenous NADP+ to utilizable levels of NADPH. Cohen and Estabrook (477) have shown that the presence of even very low concentrations of NADPH could promote a more efficient use of NADH by the mixed function oxidase (MFO) system. This resulted in a synergistic increase in product formation by microsomal hydroxylase enzymes which can occur with the concurrent accessibility of both reduced pyridine nucleotides (NADPH and NADH) (see Section 3.1.3.1.2) (58-68, 71). Synergism could conceivably account for the marked increase in hydroxylase activity presently observed with the NADH-generating system, whereas no such effect would be expeced when the commercially reduced form of NADH was used. When such was the procedure, the inclusion of G6P was not required, and accordingly, not available to generate endogenous NADPH to produce the synergistic effect.

In order to be assured that the liver preparations used in the current study were indeed able to support a synergistic mechanism, the aliphatic hydroxylation of propiophenone or phenylacetone in the presence of rat liver 10 000X*g* supernatant supplemented with varying proportions of the reduced forms of NADP and NAD was measured (Fig. 31). It was found that the oxidation of propiophenone to 2-hydroxy-1-phenyl-1-propanone did exhibit a true synergistic effect with the concurrent inclusion of NADPH and NADH (Fig. 31A). The

FIGURE 31. NADH-Synergism - The effect of simultaneous availability of NADPH and NADH on the aliphatic C-hydroxylation of: A) propiophenone (I) to 2-hydroxy-1-phenyl-1-propanone (VII); B) phenylacetone (III) to 1-hydroxy-1-phenyl-2-propanone (VI); by fortified rat liver 10 000Xg supernatant



extent of microsomal hydroxylation was enhanced to over 150% that obtained when NADPH alone was used. This greatly exceeded an additive effect of the action of the two cofactors as hydroxylation of propiophenone in the presence of NADH alone is relatively low (15-20%) compared with the use of NADPH.

An examination of Figure 31B reveals that inclusion of NADH in the NADPH-fortified rat liver preparation did not promote a significant increase in the formation of 1-hydroxy-1-phenyl-2-propanone through hydroxylation of phenylacetone. A small increase in the overall level of metabolite produced was observed when both NADPH and NADH were added, compared to the amount of product formed in the presence of NADPH alone. However, this slight enhancement was approximately equal to the degree of hydroxylation which was measured when NADH alone was used to support this reaction. Thus, in this instance the effect appears to be an additive one.

Numerous investigators have measured the concentrations of the oxidized and reduced pyridine nucleotides in various in vitro rat liver preparations including isolated organ perfusions, homogenates and 10 000X*g* supernatants, and 105 000X*g* cytosol and microsomal fractions (463, 466-469, 473, 478-480). All studies support that the major proportion of NAD occurs in these preparations as NAD+. Conversely, the majority of studies concluded that the predominant form of NADP in rat liver is in its reduced state (NADPH); only Lowry et al. (481) and Reinke et al. (480) found the opposite to be true.

The predominance of NAD+ over NADH is understandable, due to the absence in rat liver of specific NAD-linked dehydrogenases required to enzymically reduce the NAD+ (462). With NADP however, although sufficient levels of NADP-linked dehydrogenases are assumed to be present to maintain a higher proportion of NADPH than NADP+ (461), under the incubating condition used in the current study an obvious deficiency of such dehydrogenases was apparent. This conclusion could be made from data presented earlier (Table 9) which indicated that addition of a NADP-specific G6PDH to the NADPH-generating system was required for optimal metabolizing activity and in the present experiment where the lack of adequate dehydrogenases was more directly demonstrated (Table 10). Supplementing NADP-linked G6PDH in the incubation mixture containing no commercially added NADP+, markedly increased the extent of propiophenone metabolism. The greatest change occurred with the concurrent addition of G6P with the G6PDH, where the rate of reduction and hydroxylation was raised 9-10 fold and 14-21 fold, respectively. These results illustrated the availability of sufficient levels of endogenous NADP+ (and NAD+), but inadequate amounts of endogenous G6PDH (and G6P) to promote the conversion of NADPH/NADH to the active, reduced form. A logical conclusion from these studies is that under the prevailing conditions by which tissue preparations are obtained, both endogenous NADP and NADH exist primarily in their oxidized state (NADP+ and NAD+).

5.1.1.3.3.3 Mg++ Requirements

Although the divalent cation Mg++ is routinely included in incubation mixtures (usually in the form of $MgCl_2$) in order to facilitate production of NADPH/NADH, magnesium's exact association with the cofactor generating system has not been established. Several studies on glucose-6-phosphate dehydrogenases have found that whereas the majority of NADP- or NAD- linked dehydrogenases could be stimulated by the addition of Mg++, the presence of Mg++ was not an absolute requirement for catalytic activity (461, 462, 470). Other investigators have shown that with various dual nucleotide specific G6PDHs, both NADP- and NAD-linked reactions required the addition of Mg++ (474, 482). On the other hand, still others have claimed that although enhancement of the NADP-reactions did occur in the presence of Mg++, inhibition of the NAD-linked reactions was simultaneously noted (472, 483).

In the current study, no activating effect on the metabolizing system by the addition of Mg++ could be demonstrated. This was regardless of whether rat liver 10 000X*g* supernatant was the sole source of endogenous G6PDH, or if the incubation mixture was supplemented by the addition of the NADP-linked Baker's Yeast G6PDH or the dual nucleotide specific Leuconostoc mesenteroides G6PDH. It was decided however, not to eliminate $MgCl_2$ from future incubations. When activation of various G6PDHs was reported, it could only be observed at low concentrations of Mg++. Thus, even if the usefulness of $MgCl_2$ was questionable, its addition in very small amounts caused no detrimental effects to metabolic activities and its addition was continued.

In addition to Mg++, other divalent or monovalent cations (Ca++, Mn++, K+, Cu++, and others) have been reported to activate various types of G6PDHs, depending on their source (484-487). No thorough attempt, however, was made to investigate completely the requirements for these ions.

5.1.1.3.3.4 Nicotinamide

An early problem apparently observed in in vitro microsomal mixed-function oxidase systems had been the rapid breakdown of NADP+ by endogenous pyridine nucleotidases. This problem was particularly prevalent at low concentrations of cofactor. As a result, in order to prevent the destruction of NADP+ and assure maximum microsomal activity, the addition of nicotinamide as a competitive substrate for the nucleotides was recommended (60). Although a similar addition of nicotinamide has not been suggested for optimum reductase activity, the same beneficial effect would be expected by maintaining appropriate levels of NADP/NAD.

Thus, it was important to learn what influence the presence of nicotinamide would have on the in vitro metabolism of propiophenone (I) and phenylacetone (III).

Examination of the data presented in Table 9, shows that supplementation of the incubation mixtures with 30 umol of nicotinamide caused a slight decline in the degree of aliphatic hydroxylation of both propiophenone (I) and phenylacetone (III), regardless of the cofactor used. The same result was found also with the extent to which these ketone— containing compounds (I, III) underwent metabolic reduction. Neither effect was completely unexpected though. Several studies have demonstrated that the presence of nicotinamide under certain conditions interferes with rates of metabolism (488-490). Since nicotinamide itself has been observed to undergo N-oxidation in vitro (491), there is some suggestion that the interference is of a competitive nature (490). This was partially demonstrated in the present study where the decreases in the extent of reduction and hydroxylation caused by the addition of nicotinamide could be offset slightly by increasing the concentration of cofactor (NADP/NAD) from 4.4 umol to 8.8 umol. A comparable increase in the degree of metabolism was not observed when the cofactor amount (4.4 umol) of the standard incubation mixture, containing no nicotinamide, was also increased to 8.8 umol. Since supplementation with nicotinamide had no obvious benefits to in vitro metabolic systems, the practice was not continued in further studies.

5.1.1.3.3.5 Isocitrate Dehydrogenase

In conjunction with studies which utilized a G6PDH-linked NADPH/NADH-generating system, an alternative pathway for the production of the reduced form of coenzymes was also evaluated. Numerous investigators have relied on isocitrate dehydrogenase (ISCDH) to catalyze the reduction of NADP+ to NADPH (Fig. 30) and, as might be expected, wide variations in the incubation preparation were apparent in these investigations. The ISCDH system utilized in the current study was based on such published data.

As indicated by the results presented in Table 9, the ISCDH system worked well for the generation of NADPH. Substitution of NADP+ with NAD+, however, led to an almost complete loss of metabolic activity. This appeared to be the result of two distinct isocitrate dehydrogenases found in rat liver tissue; one which was dependent solely on the availability of NADP, and the other solely on NAD (31). But whereas the NADP-linked ISCDH is highly active and has a significant role in the oxidation of isocitrate, the role of the NAD-dependent dehydrogenase is as yet unclear and does not appear to be associated with the reduction of NAD+ (491, 492).

Despite reports that rat liver 10 000X$\underline{g}$ supernatant normally contains an excess level of endogenous NADP-linked isocitrate dehydrogenase (461), maximal generation of NADPH could only be achieved by supplementing the incubation mixture with porcine heart ISCDH (12 units). The requirement for a commercial source of an NADP-linked ISCDH indicated a deficient endogenous level of this enzyme. Unfortunately, a commercial product of an NAD-dependent dehydrogenase could not be similarly obtained, thus eliminating the possibility of using an auxiliary ISCDH system for the in vitro production of NADH. As this presented a major barrier to further studies comparing cofactor differences, studies on the use of the ISCDH generating system were discontinued.

5.1.1.3.4 Cofactor Concentrations

Apart from the ketone reductase and mixed function oxidase systems, hepatic tissue also contains numerous other enzymes that catalyze reactions requiring reduced pyridine nucleotides. If metabolized by these alternative metabolic routes, both endogenous and foreign compounds could potentially compete for available NADPH and for NADH. Liver 10 000X$\underline{g}$ supernatants also contain NAD-glucohydrolase and nucleotide pyrophosphatase enzymes which degrade pyridine nucleotides and could also lessen the availability of both NADH and NADPH for biotransformations. As a consequence of these possibilities, efforts were made to assure that the concentrations of added cofactors were sufficient enough so as to not constitute a limiting factor and restrict product formation, particularly at higher substrate concentrations.

Levels of metabolites obtained from incubating propiophenone (I) or phenylacetone (III) with rat liver 10 000X*g* supernatant in the presence of increasing amounts of NADPH or NADH (1-10 umol) were measured. The commercially reduced forms of the coenzymes were used in order to eliminate any limiting conditions caused by a possibly ineffective cofactor generating system.

Even at a substrate concentration (1.0 umol) higher than that used in most studies, maximum metabolite formation was obtained following supplementation with 2.8 umol cofactor (NADPH or NADH). Higher cofactor levels did not further increase metabolism. As a further test the same experiment was duplicated using rabbit liver 10 000X*g* supernatant, because of its demonstrated greater capacity for the *in vitro* metabolism of the substrates (I, III) than was possible with rat liver (Section 5.1.1.2.1). Under these conditions, levels of metabolites reached maximum production after fortification of the incubation mixture with 3.5 umol of cofactor. Thus, regardless of the species used as the source of tissue, limiting cofactor concentrations were found to be below that of the 4.4 umol added in the standard incubation mixture. This information confirmed that adequate amounts of cofactors (NADPH, NADH) were being used to guarantee full drug metabolizing enzymic activity.

5.1.1.4 Properties of Ketone Reductases Present in the 10 000X*g* Supernatants of Rat and Rabbit Liver Homogenates

Several mammalian NADP- and/or NAD-linked enzymes which are capable of reducing xenobiotic ketonic compounds have been characterized by various investigators (see Section 3.1). Although a few reductases have been purified prior to use, the majority have been studied either in unfractionated tissue homogenates, or as partially purified enzyme mixtures. As the current investigation was concerned essentially with properties of the intact ketone reductase system, no attempt was made to isolate the enzymes from their complex environment.

Several general traits pertaining to substrate and cofactor specifities as exhibited by ketone reductases present in rat and rabbit liver preparations have already been reported in preliminary investigations (Section 5.1.1.2.2). The following course of study expands on many of the properties demonstrated by these reductases.

5.1.1.4.1 Reductase Subcellular Location

The data presented in Table 11 indicates that almost all of the demonstrated mammalian ketone reductase activity

TABLE 11. Subcellular Localization of Enzymes Responsible for Metabolic Carbonyl Reduction.

	REDUCTION (%)			
	PROPIOPHENONE		PHENYLACETONE	
TISSUE (LIVER) FRACTION [a]	NADPH	NADH	NADPH	NADH
WHOLE SUPERNATANT (10 000Xg HOMOGENATE)	26	48	4	50
MITOCHONDRIAL	TR[b]	TR	--	TR
MICROSOMAL (105 000Xg PELLET)	1	3	TR	3
CYTOSOL (SOLUBLE) (105 000Xg SUPERNATANT)	16	32	3	37

[a] RAT LIVER TISSUE

[b] TR < 0.2%

was found to occur within the cytosol fraction (105 000X*g* supernatant), and little or no reductase activity could be located in either the microsomal (105 000X*g* resuspended pellet) or mitochondrial fractions.

Localization in the cytoplasm is one of the prevalent features which discriminates carbonyl reductases (both ketone and aldehyde) from most other drug-metabolizing enzymes (12, 13, 40, 494). There does appear, however, to be exceptions to this rule. Several NAD- or NADP-linked microsomal and mitochondrial reductases have been reported (120, 494-496) including oxisuran reductases which have been associated with the insoluble mitochondrial membrane, as well as the soluble (cytosol) fraction (497).

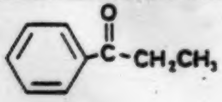
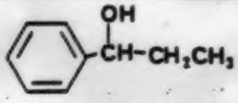
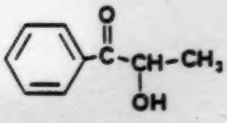
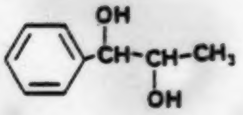
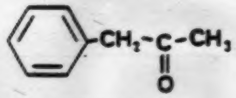
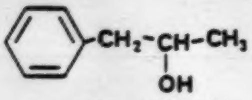
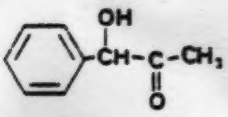
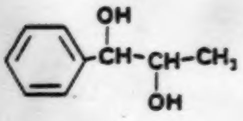
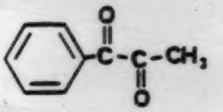
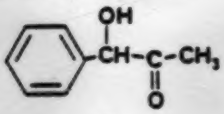
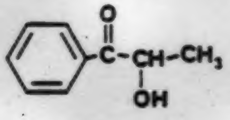
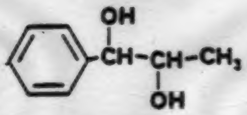
Since carbonyl reductases are categorically oxidoreductases, the same enzymes should also be capable of catalyzing the oxidation of primary and secondary alcohols. The efficiency of these reverse dehydrogenation reactions are discussed in a later section (Section 5.1.1.4.5), but in those instances where the oxidation of an alcohol was observed, dehydrogenase activity was located only in the cytosol fraction. Reversibility of the oxidative reaction was not detected in either the microsomal or mitochondrial fractions prepared from rat or rabbit liver.

5.1.1.4.2 Reductase Substrate/Cofactor Specificity

Additional studies which further demonstrated substrate and cofactor specificities of reductases located in mammalian tissue were carried out. Several analogues of propiophenone and phenylacetone were reduced in the presence of rat or rabbit 10 000X*g* liver supernatant fortified with either NADPH or NADH. As illustrated in Table 12, there are marked differences between the capability of the two species to reduce ketone-containing compounds. In general, rabbit liver possesses a more efficient reductase system, regardless of the nature of the substrate or cofactor. Aliphatic ketones were reduced to a slightly greater extent than the analogous aromatic ketones, and NADPH was a moderately more efficient cofactor than NADH. This contrasts with conclusions reached by others (40, 42, 493) that the reduction of aromatic ketones is specifically NADPH dependent, and aromatic ketones are preferentially reduced over aliphatic ketones (12, 28, 42). With reduction by rat liver 10 000X*g* supernatant however, no generalizations could be made. Reduction varied significantly with changes in both substrate and cofactor, possibly indicating that the reduction of aromatic and aliphatic ketones were mediated by more than one enzyme in rat liver cytosol.

In view of these findings, absolute substrate specificity of particular enzymes was difficult to assess, as

TABLE 12. Metabolic Ketone Reduction by Fortified Rat and Rabbit Liver 10 000X*g* Supernatant, as a function of Cofactor and Substrate Specificity.

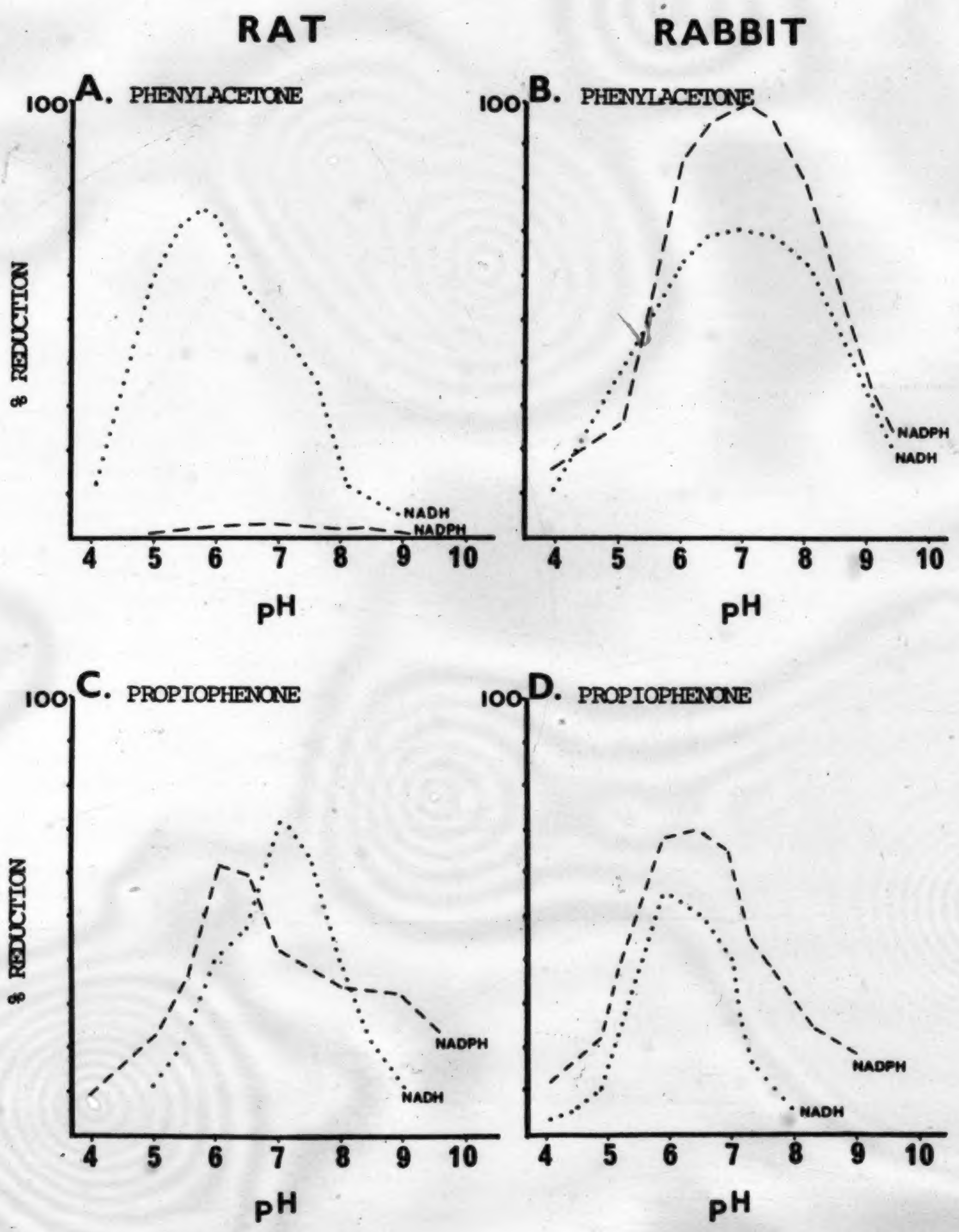
SUBSTRATE	PRODUCT	REDUCTION (%)			
		RAT		RABBIT	
		NADPH	NADH	NADPH	NADH
 PROPIOPHENONE		26.6 ± 1.85	48.5 ± 3.20	65.3 ± 3.33	40.4 ± 2.71
 2-HYDROXY-1-PHENYL-1-PROPANONE		38.2 ± 2.20	28.1 ± 2.01	85.1 ± 3.41	73.3 ± 2.95
 PHENYLACETONE		3.81 ± 0.32	51.8 ± 2.03	92.3 ± 4.02	81.6 ± 3.57
 1-HYDROXY-1-PHENYL-2-PROPANONE		65.3 ± 3.25	55.5 ± 3.11	90.2 ± 4.30	81.7 ± 3.51
 1-PHENYL-1,2-PROPANEDIONE		9.01 ± 0.86	17.2 ± 0.98	2.90 ± 0.18	2.00 ± 0.15
		33.7 ± 1.87	40.8 ± 2.36	18.3 ± 1.21	23.7 ± 0.76
		36.5 ± 1.03	24.4 ± 1.32	72.5 ± 2.54	60.0 ± 2.19
	TOTAL	79.2 ± 2.85	82.4 ± 3.01	93.7 ± 3.99	85.7 ± 4.14

cytoplasmic extracts or crude liver supernatants may actually contain several reductases. In order for a more detailed description of substrate specificity to be carried out it would be necessary to rely on the purified forms of these enzymes.

5.1.1.4.3 Reductase pH Optima

The pH profiles of metabolic reductions by rat or rabbit liver preparations fortified with either NADPH or NADH were examined. The extent to which reduction of propiophenone (I) and phenylacetone occurred was measured over the range pH 4-10 in Tris-KCl buffer (Fig. 32). In all instances, an acidic optimum was observed, regardless of species, substrate, or cofactor. Only slight reductase activity was detected below pH 4 or above pH 10. These results were consistent with the findings of Felsted and Bachur (498) who reported that although aldehyde and ketone reductases normally have pH optima ranging between pH 5.0 and 8.5, most enzymes operated best within a range of pH 5.5-7.0. When incubated in the presence of rabbit 10 000X*g* supernatant, maximum reductase rates were detected at pH 6.0 for propiophenone and at pH 6.8 for phenylacetone (Figures 32D and 32B). Similar pH profiles were observed when either NADPH or NADH was utilized as the coenzyme. With rabbit liver preparations, a broad pH optimum for both substrates was displayed.

FIGURE 32. Phenylacetone and propiophenone reductase activity - pH profiles of rat and rabbit liver 10 000X*g* supernatants.



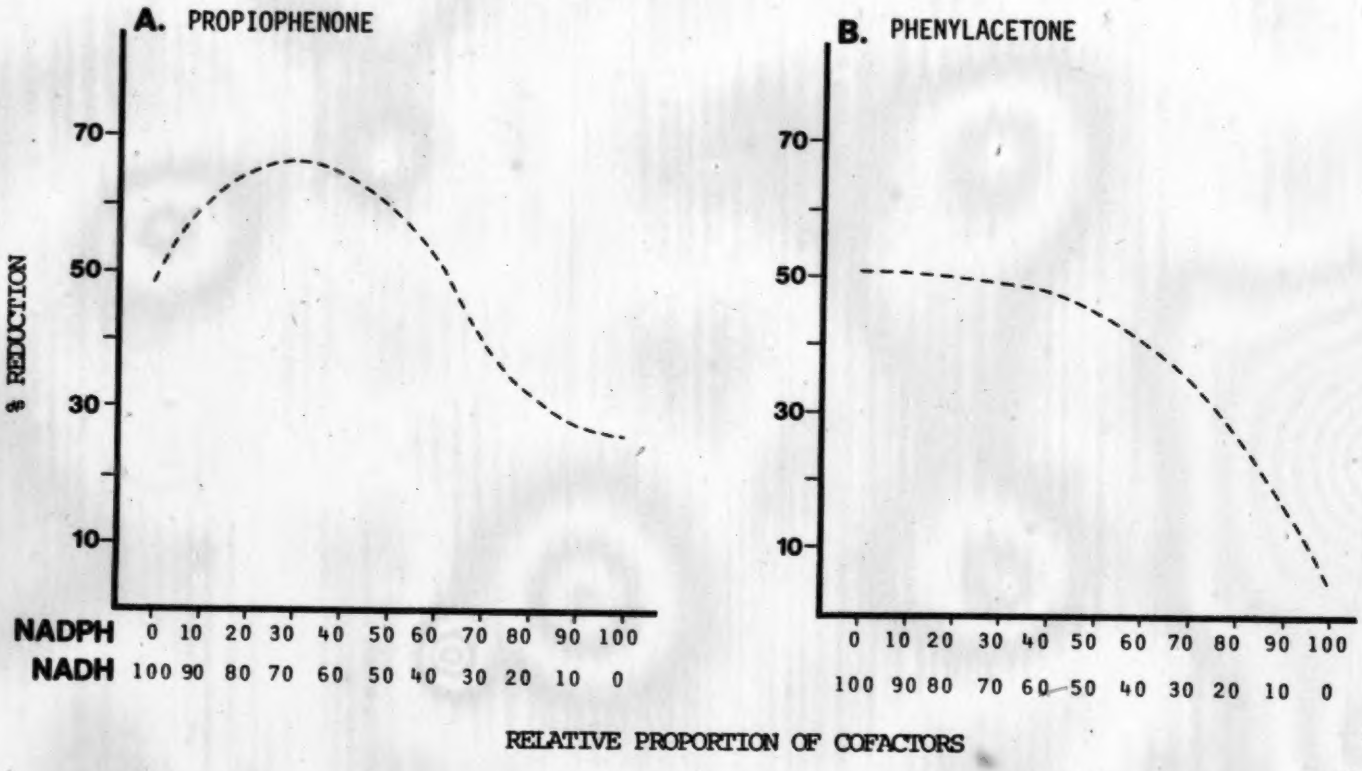
Maximum activity for the reduction of phenylacetone by NADH-fortified rat liver supernatant occurred at a slightly lower pH (5.8) than what was observed with rabbit liver preparations (Fig. 32A). No optimum pH could be determined when an NADPH-fortified preparation was utilized. Reduction of propiophenone (I) in vitro by rat resulted in two distinct pH optima, depending on the cofactor employed (Fig. 32C). This suggested that at least two distinct reductases, capable of reducing this aromatic ketone (I), were present in rat liver. Maximum activity was evident at pH 6.0 for the NADP-linked reductase, and somewhat higher (pH 7.0) for the NAD-dependent reductase. No other example of an NAD-specific aromatic ketone reductase appears to have been reported, although several NAD-linked aromatic/aldehyde reductases have been isolated which displayed very slight reactivity towards aromatic ketones (232, 289, 362, 379). These enzymes were quite different from the classical NAD-dependent pyrazole-sensitive alcohol dehydrogenase (ADH), and are not considered true ketone reductases.

5.1.1.4.4 Reductase NADH-Synergism

It has already been suggested that a synergistic enhancement of the mixed function oxidase (MFO) system could occur if both NADP and NAD, in their reduced states (NADPH, NADH), were made available (Section 5.1.1.2.3.2). While the mechanism of metabolic reduction is undeniably different from that of microsomal hydroxylation, both processes do rely on the acceptance of two reducing equivalents to complete the reaction. It was of interest therefore, to determine whether a similar synergistic effect would be observed with the ketone reductase system in the presence of both cofactors. This might possibly provide some insight into the source of the second reducing equivalent required during reduction.

The reduction of propiophenone or phenylacetone by rat liver 10 000X*g* supernatant fortified by varying proportions of NADPH and NADH was monitored (Fig. 33). The information presented in Fig. 33B shows that concurrent addition of NADPH did not increase the level of the NADH-mediated reduction of phenylacetone. Maximum activity was observed with 100% NADH, whereas increasing the proportion of NADPH effectively decreased the overall extent of metabolic reduction. With propiophenone however, reduction did increase marginally with the availability of both cofactors. The highest level of reduction (65%) was obtained at a cofactor ratio of approximately 70/30 (NADH/NADPH). While this was greater than with either coenzyme separately, the effect was neither synergistic nor completely additive (Fig. 33A). This outcome might have been expected based on information obtained from an earlier study (Section 5.1.1.4.3) which suggested that propiophenone could be reduced by either of two cofactor-distinct enzymes found in rat liver. With the simultaneous addition of both cofactors to the incubation mixture, an

FIGURE 33. NADH-Synergism - The effect of simultaneous availability of NADPH and NADH on the carbonyl reduction of: A) propiophenone (I) to 1-phenyl-1-propanol (II); B) phenylacetone (III) to 1-phenyl-2-propanol (IV); by fortified rat liver 10 000Xg supernatant.



NADP- dependent reductase and an NAD-dependent reductase were each able to separately contribute to the total product formed.

5.1.1.4.5 Metabolic Dehydrogenation of Alcohols

If the enzyme systems which are present in 10 000X*g* supernatant of homogenized rat and rabbit liver and which have been shown to be capable of catalyzing the reduction of propiophenone and phenylacetone are in fact oxidoreductases (12, 19), these systems should also be capable of oxidizing (*i.e.* dehydrogenating) the reduced forms of these ketones (1-phenyl-1-propanol and 1-phenyl-2-propanol, respectively) back to the corresponding ketones. This oxidative property was observed (Table 13). The extent of dehydrogenation varied with both substrate and species. Oxidation of 1-phenyl-2-propanol to phenylacetone occurred to only a minor extent regardless of whether a rat or rabbit liver preparation was used, or whether NADP or NAD was the cofactor employed. Dehydrogenation of 1-phenyl-1-propanol to propiophenone also occurred to only a small degree with rabbit liver 105 000X*g* supernatant fortified with either an NADPH- or NADH-generating system. A significantly greater amount (16-18%) of propiophenone was produced when an $NADP^+$ or NAD^+ fortified rat liver preparation was utilized. This formation of propiophenone from 1-phenyl-1-propanol was an example of the metabolic oxidation of an α-arylalcohol to the

TABLE 13. The Extent of *In Vitro* Metabolic Dehydrogenation (by liver 105 000X*g* supernatant) of Various Alcohol Substrates, as a function of Cofactor and Species.

SUBSTRATE	PRODUCT	% DEHYDROGENATION			
		RAT		RABBIT	
		NADP+	NAD+	NADP+	NAD+
1-PHENYL-1-PROPANOL	PROPIOPHENONE	16.1	17.8	4.4	8.2
1-PHENYL-2-PROPANOL	PHENYLACETONE	7.1	6.8	1.5	5.4
1-HYDROXY-1-PHENYL-2-PROPANONE	1-PHENYL-1,2-PROPANEDIONE	TR.[1]	TR	ND.[2]	ND.
2-HYDROXY-1-PHENYL-1-PROPANONE	1-PHENYL-1,2-PROPANEDIONE	TR.	TR.	ND.	ND.
1-PHENYL-1,2-PROPANEDIOL	1-HYDROXY-1-PHENYL-2-PROPANONE	ND.	ND.	ND.	ND.
	2-HYDROXY-1-PHENYL-1-PROPANONE	ND.	ND.	ND.	ND.
	1-PHENYL-1,2-PROPANEDIONE	ND.	ND.	ND.	ND.

[1] TR. < 0.2%

[2] ND. - NOT DETECTED

corresponding ketonic compound. In a recent review, Testa and Jenner (11) claimed that no examples of metabolic dehydrogenation of α-arylalcohols to aromatic ketones were known, but in fact, several have been reported. Liebman (493) observed that 1-phenylethanol could be oxidized to acetophenone with either NADP+ or NAD+ fortified rabbit liver cytosol. Culp and McMahon (42) have reported that p-methoxylbenzyl alcohol was slowly oxidized at a high pH (10—11) in the presence of an $NADP^+$-30 000Xg rabbit kidney supernatant preparation, although there was no evidence of oxidation at physiological pH (7.4). The report by Sinsheimer *et al.* (460) that the *in vitro* metabolism of norephedrine with rabbit liver homogenates yielded 1-phenyl-1,2-propanedione as a minor metabolite also indicated that dehydrogenation of a benzylic alcohol group had occurred.

Not all metabolic reactions involving an oxidoreductase enzyme are reversible, however. The reversibility of such reactions appeared to be due in part to the nature of the alcohol substrate, as well as the reacting enzyme. For instance, metabolic oxidation could not be demonstrated with several close analogs of 1-phenyl-1-propanol (II), although II itself was substantially oxidized in the presence of fortified rat liver 105 000Xg supernatant (Table 13). The dehydrogenation of 2-hydroxy-1-phenyl-1-propanone (VII), an analog of 1-phenyl-1-propanol (IV), was also unsuccessful. Felsted and Bachur (12) however, have stated that very few reverse oxidations are observed below pH 8-9. This probably accounted for the low or absent dehydrogenation activity detected with the substrates tested in the current study. The oxidation of 1— phenyl-1-propanol by fortified rat liver supernatant appeared to be the exception.

5.1.2 Stereoselectivity of Metabolic Reduction

Metabolic reduction of an unsymmetrical ketone containing compound will yield an alcohol possessing an asymmetric center. As greater numbers of biological occurrences are being explained in terms of molecular interactions, it was of interest to examine the relationship between metabolic reduction and its stereochemical significance. It has been described previously how several ketones undergo a stereoselective reduction by mammalian reductases (Section 3.1.3.2.3). In the current study, the metabolic reduction of three arylalkylketones (propiophenone, I; phenylacetone, III; and 1-phenyl-1,2-propanedione, V), and procedures which allowed effective separation of the recovered stereoisomeric alcohols (*RS*-1-phenyl-1-propanol, II; *RS*-1-phenyl-2-propanol, IV; and *erythro*/*threo*-1-phenyl-1,2-propanediol, VIII) were investigated. *In vivo* and *in vitro* metabolic reductions were studied in two species and a comparison of the extents to which stereoselectivity occurred is provided.

5.1.2.1 Resolution of Stereoisomers

To determine the degree of stereoselectivity, a reliable method was required for the resolution of alcohol stereoisomers. Conventional methods for determining the optical purity of metabolites have generally relied on isolating sufficient quantities of the product for polarimetric analysis (102, 118, 499-501). With many metabolic studies, however, the amount of product available for stereochemical analyses is often very small. This makes isolation and resolution procedures, particularly the accurate measurement of optical rotation, extremely difficult. The use of chiral reagents and subsequent analysis of diastereoisomeric products by GLC (502-507) offered a very sensitive alternative procedure for the quantitation of stereoisomeric metabolites.

Initially, it was necessary to determine the GLC elution sequence of the mixtures of diastereosiomers formed by the reaction of the racemate alcohols (II and IV) with the chiral reagents [S(+)-α-methylbenzyl isocyanate, MBIC; R(-)-menthylchloroformate, MCF]. To accomplish this, authentic samples of II and IV were synthesized by a procedure which was known to produce the alcohols enriched in one of the enantiomers. 1-Phenyl-1-propanol (II) was prepared by the asymmetric reduction of propiophenone (I) with a sodium L-proline borane complex to yield a mixture of the enantiomers in which the S(-)-isomer predominated. A comparison of the magnitude of the optical rotation of the mixture with that of a pure sample of S-(-)-1-phenyl-1-propanol permitted the calculation of the amounts of S(-)-II and R(+)II in the synthetic product (Table 14). 1-Phenyl-2-propanol (IV) was prepared from phenylacetone (III) in a similar manner. The mixture of stereoisomers was strongly dextrorotatory, indicative of S(+)-IV being in enantiomeric excess. The calculated ratio of S(+)-IV/R(-)-IV is illustrated in Table 14.

The mixture of stereoisomers, RS-II or RS-IV were then reacted with the chiral reagents (MBIC and MCF) and the products analyzed by GLC (Fig. 34). The inability to detect any unreacted alcohols confirmed complete derivatization. Peak areas were integrated and the ratio of isomers calculated. The values obtained were in close agreement with those determined from polarimetric data (Table 14), thus permitting peak identification.

The efficiency of peak resolution varied. A superior separation of R- and S-1-phenyl-2-propanol (IV) was achieved after derivatization with S(+)-α-methylbenzyl isocyanate (MBIC) than with R(-)-menthyl chloroformate (MCF) (Fig. 34). This resulted in a more precise determination of optical purity using MCF (Table 14). With R- and S-1-phenyl-1-propanol (II) though, better GLC separation was obtained following derivatization with MCF than with MBIC. However, the calculation of isomer ratios from the two chromatograms

TABLE 14. The Stereochemical Analysis of Alcohols Obtained by Chemical Reduction of Arylalkylketones. A Comparison of the Ratio of Enantiomers Detected by Different Analytical Methods.

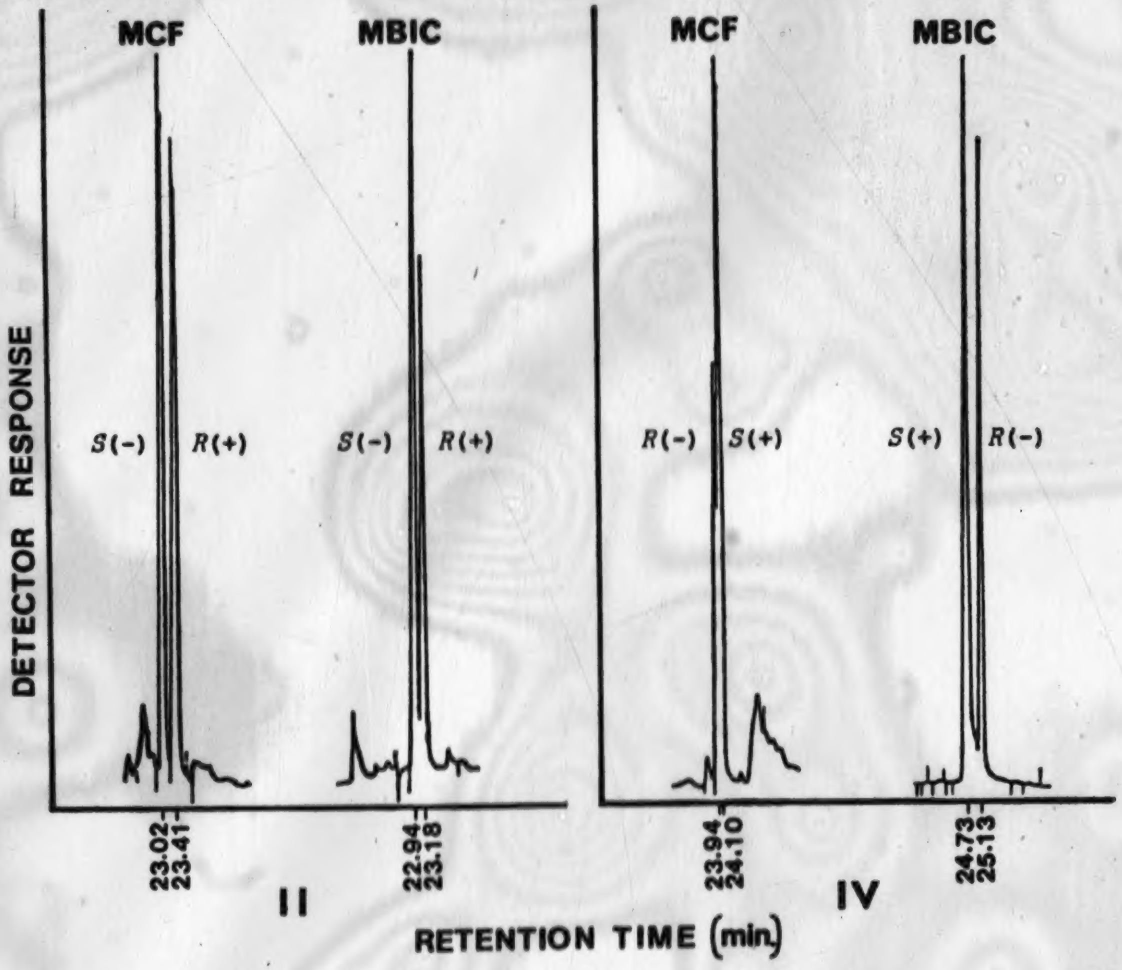
METHOD OF ANALYSIS	ALCOHOLS [a]	
	II	IV
POLARIMETRIC	72 / 28 [b]	60 / 40 [c]
DERIVATIZATION / GLC		
R(-)-menthyl chloroformate	71 / 29	65 / 35
S(+)-α-methylbenzyl isocyanate	72 / 28	58 / 42

[a] RATIO OF ENANTIOMERS: 1-PHENYL-1-PROPANOL (II), *S*(-)/*R*(+)
1-PHENYL-2-PROPANOL (IV), *S*(+)/*R*(-)

[b] BASED ON $[\alpha]_D$-32.50 (c=5.1, EtOH). Pickard and Kenyon (440).

[c] BASED ON $[\alpha]_D$+16.10 (c=5.6, EtOH). Pickard and Kenyon (440).

FIGURE 34. Capillary gas liquid chromatograms of the diastereoisomeric pairs formed by the reaction of 1-phenyl-1-propanol (II) and 1-phenyl-2-propanol (IV) with chiral derivatizing reagents. [MCF = *R*(-)-menthyl chloroformate, MBIC = *S*(+)-α-methylbenzyl isocyanate]



were virtually identical and in excellent agreement with those obtained polarimetrically (Table 14).

5.1.2.2 Stereoselective Reduction of Ketones

5.1.2.2.1 Propiophenone and Phenylacetone

Having illustrated that the enantiomers of the racemate alcohols (II and IV) could be efficiently separated and quantitated as the corresponding diastereoisomers, it was possible to determine the extent to which stereoselectivity occurred during the metabolic reduction of arylalkylketones (I, III) and to assign absolute configuration to the products. Data summarized in Table 15 reveals that the metabolic reduction of propiophenone (I) and phenylacetone (III) demonstrated a high degree of product stereoselectivity. The metabolites, 1-phenyl-1-propanol (II) and 1-phenyl-2-propanol (IV) respectively, were recovered predominantly as their *S*-isomers in all studies with one exception; *in vivo* reduction of I by rat produced approximately equal amounts of the *R*- and *S*-isomers.

5.1.2.2.1.1 *In Vitro* Reduction

It was observed that when propiophenone (I) was reduced *in vitro*(10 000X*g* liver homogenate) by rat or rabbit, 93-97% of the product alcohol (II) occurred as the *S*(-)-isomer, the remainder being the *R*(+)-form (Table 15). Species differences were minimal and cofactor, NADPH or NADH were equally efficient. A structurally related alcohol, 1-phenyl-

TABLE 15. The Stereochemical Analysis of Alcohols obtained from the Metabolic Reduction of Arylalkylketones.

METHODOLOGY					RATIO of ENANTIOMERS of METABOLIC ALCOHOLS [c]	
SUBSTRATE	SYSTEM	COFACTOR[a]	PRETREATMENT[b]	METABOLIC ALCOHOL	SPECIES	
					Rat	Rabbit
I	*IN VITRO*	NADPH	N/P	II	96 / 4	93 / 7
		NADH	N/P		97 / 3	94 / 6
	IN VIVO	—	N/P		57 / 43	91 / 9
		—	ENZYME		52 / 48	89 / 11
		—	ACID		42 / 58	65 / 35
III	*IN VITRO*	NADPH	N/P	IV	91 / 9	94 / 6
		NADH	N/P		90 / 10	92 / 8
	IN VIVO	—	N/P		81 / 19	87 / 13
		—	ENZYME		80 / 20	88 / 12
		—	ACID		72 / 28	79 / 21

[a] GENERATING SYSTEM; GLUCOSE-6-PHOSPHATE, $MgCl_2$, and NADP+ or NAD+.

[b] HYDROLYTIC PRETREATMENT OF BIOLOGICAL SAMPLE; N/P -NO PRETREATMENT, ENZYME -β-GLUCURONIDASE (15 000 *U*/ml, INCUBATED 36 Hr. at 37°), ACID -pH<1 (AUTOCLAVED 40 Min. at 125° and 15 *psi*.)

[c] RATIO OF ENANTIOMERS (as DIASTEREOISOMERIC DERIVATIVES); 1-PHENYL-1-PROPANOL [*S*(-)/*R*(+)-II] as *R*(-)-MENTHYL CHLOROFORMATE DERIVATIVE, 1-PHENYL-2-PROPANOL [*S*(+)/*R*(-)-IV] as *S*(+)-α-METHYLBENZYL ISOCYANATE DERIVATIVE.

1-ethanol, produced by the reduction of acetophenenone in the presence of a rabbit liver preparation (507) or a rabbit kidney preparation (42) was similarly found to be predominantly in the S(-)-form (97% and 76% respectively).

In vitro reduction of phenylacetone (III) in rat and rabbit coincided with that observed with substrate I. The alcohol, 1-phenyl-1-propanol (IV), was recovered mainly as the S(+)-enantiomer (90-94%), regardless of species or cofactor (Table 15). Although the extent of *in vitro* reduction of arylalkylketones was shown in earlier studies (Section 5.1.1.3.2) and by others (13, 42, 298, 457, 493, 508, 509) to be highly dependent on both species and cofactor, the present study indicated that enantioselective preference was virtually unaffected by these variables. This conclusion was also supported by Bently (510) who deduced several generalizations about the stereospecificity of enzymic reactions. These were, i) that the stereospecificity of a particular reaction is independent of the source of the enzyme which catalyzes it, ii) that when an enzyme can use either NAD+ or NADP+ (or their reduced forms) the stereospecificity of the reaction is the same with both coenzymes, and iii) that when an enzyme reacts with a range of substrates, the stereospecificity of the hydrogen transfer is the same with each substrate.

5.1.2.2.1.2 In Vivo Reductions

If Bently's (510) generalizations reproduced above were indeed correct, a strong correlation between in vitro and in vivo studies would therefore be expected. In the current investigation this was indeed shown to hold true for both substrates (I, III) when utilizing rabbit (Table 15). A good correlation was also demonstrated between in vivo and in vitro reduction of phenylacetone (III) in the rat, but not when propiophenone (I) was the substrate. In the latter instance, enantioselectivity was clearly demonstrated in in vitro studies, but in vivo reduction of I appeared to be non-stereoselective.

In vivo reduction of propiophenone (I) by rabbit gave results comparable to that observed in the corresponding in vitro studies (Table 15). Approximately 90% of the alcohol recovered from urine was determined to be S(-)-1-phenyl-1-propanol [S(-)-II]. An earlier study by Smith et al. (102) indicated that following administration of propiophenone (I) to rabbits, its metabolite II was isolated entirely as the S(-)-glucuronide conjugate.

In vivo reduction of phenylacetone (III) to its corresponding alcohol 1-phenyl-2-propanol (IV), also resulted in the preferential formation of the S-isomer [S(+)-IV] (Table 15). The ratio of enantiomers of IV [S(+)/R(-)] formed by rat and rabbit in vivo were 81/19 and 87/13 respectively, which corresponded to approximately twice the amount of R(-)-

IV produced in vitro In agreement, a previous study indicated that when phenylacetone (III) was orally administered to rabbit, the S-isomer of 1-phenyl-2-propanol (IV) was preferentially produced (114). In fact, no trace of R(+)-IV could actually be found.

The one anomalous situation was the apparent loss of stereoselectivity observed with the in vivo reduction of propiophenone (I) in rat; this was suspected to be due to the further preferential metabolism of the S(-)-isomer of the resulting alcohol metabolite, 1-phenyl-1-propanol (II). A related investigation by McMahon and Sullivan (511) involving an analog of II, supported this suggestion. They established that when 1-phenyl-1-ethanol was administered to rats as the R(+)-isomer it underwent extensive conjugation and was excreted largely as the glucuronide, whereas the S(-)-isomer was further oxidized to S(-)-mandelic acid. In order to determine if similar circumstances could have accounted for the loss of product stereoselectivity observed with the metabolic reduction of propiophenone in vivo in rat, the same study as above was duplicated in which the ketone substrates (I, III) were replaced by the corresponding alcohols (II, IV). By this procedure, it was possible to determine whether the product alcohol indeed underwent some sequential stereoselective change in vivo.

The two alcohols under investigation were separately administered orally as racemic mixtures of isomers to both rat and rabbit. Isolation and stereochemical analysis of the excreted alcohol substrates were again carried out in the same manner as previously used.

The results given in Table 16 show that following oral administration of 1-phenyl-1-propanol (II) in a known isomeric ratio [49 S(-)/51 R(+)], its recovery as a urinary metabolite showed a marked decrease in the relative proportion of the S-isomer, regardless of species. By far the most significant loss of the S-II isomer occurred in rat, where the ratio of isomers of the recovered alcohol (II) was calculated to be only 15% as the S(-) isomer and 85% in the R(+) form. The change in the ratio of isomers following treatment of rabbits was less dramatic. These results strongly indicated that the apparent complete loss of stereospecificity detected during *in vivo* metabolic reduction of propiophenone (I) in rat did not actually take place during reduction, but was the result of subsequent *in vivo* interactions which occurred preferentially with the S(-)-isomers of 1-phenyl-1—propanol (II).

The ratios of isomers isolated from the *in vivo* studies were shown to vary somewhat depending on the nature of the hydrolytic treatment of the urine samples prior to extraction and derivatization of the alcohol metabolites (Table 15). A noticeable increase in the amount of R(+)-II recovered, relative to that of S(-)-II, was observed in both species after enzymic hydrolysis (Table 15). Since it has

TABLE 16. The Stereochemical Re-analysis of Arylalkylalcohols Recovered following Their *In Vivo* Administration; as a means of determining if either of the two Enantiomers of Chiral Alcohols undergoes further Preferential Metabolism *In Vivo*.

METHODOLOGY			RATIO of ENANTIOMERS of RECOVERED ALCOHOLS [a]	
SUBSTRATE (ALCOHOL)	RATIO of ENANTIOMERS [a]	PRETREATMENT [b]	SPECIES	
			RAT	RABBIT
II	49 / 51	N/P	15 / 85	43 / 57
		ENZYME	12 / 88	39 / 61
IV	52 / 48	N/P	37 / 63	44 / 56
		ENZYME	38 / 62	40 / 60

[a] ADMINISTERED RATIO OF ALCOHOL ENANTIOMERS:
II (1-phenyl-1-propanol) - 49/51 *S*(-)/*R*(+),
IV (1-phenyl-2-propanol) - 52/48 *S*(+)/*R*(-).

[b] HYDROLYTIC PRETREATMENT OF BIOLOGICAL SAMPLE:
N/P - No Pretreatment,
ENZYME - β-Glucuronidase (15 000 *U*/ml, incubated 36 Hr. at 37°C).

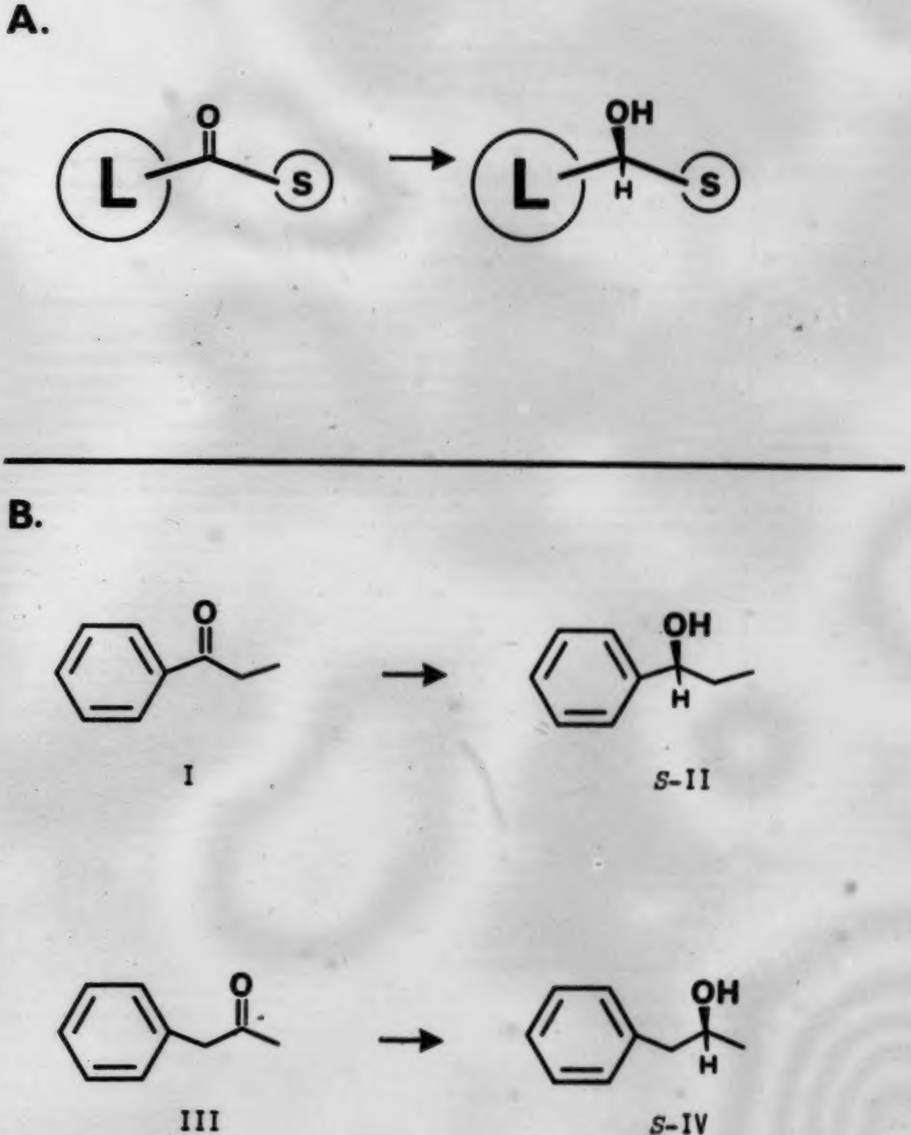
already been demonstrated that both enantiomers of II undergo glucuronide conjugation in rabbit (114), this observation suggests that a greater portion of the R(+)-isomer is conjugated relative to the S(-)-form. Only minor changes were detected in the S(+)/R(-) ratio of 1-phenyl-2-propanol (IV) after hydrolysis with β-glucuronidase when compared to the ratio obtained with no pre-treatment of the urine samples.

The much higher levels of R(+)-II and R(-)-IV measured after acidic hydrolysis could possibly have been due to a more efficient cleavage of the conjugate with acid treatment than with β-glucuronidase. However, a more plausible explanation is that racemization of isomers occurred during the harsh treatment created by conditions of low pH and elevated temperature (499). This would also account for the difficulty in obtaining reproductible results following acidic hydrolysis.

5.1.2.2.1.3 Product Stereoselectivity - Empirical Predictions

The enantioselectivity of the metabolic reduction of the arylalkylketones (I, III) could be rationalized, using the formula established by Baumann and Prelog (113) (Fig. 35A). This formula essentially stated that if the ketone containing structure is viewed with the larger substitutent placed to the left, reduction mainly occurs such that the

FIGURE 35. A) Prelog's rule of preferred product stereoselectivity. B) Predicted configuration of alcohols produced by the metabolic reduction of ketones propiophenone (I) and phenylacetone (III); based on Prelog's rule.


A.
L
O
S
OH
H
B.
O
OH
H
I
S-II
O
OH
H
III
S-IV

resultant hydroxyl group rises above the plane of the molecule. Propiophenone (I) and phenylacetone (III) could therefore, be predicted to have been preferentially reduced to alcohols with an S-configuration (Fig. 35B).

5.1.2.2.2 1-Phenyl-1,2-propanedione

In vivo and in vitro metabolism of 1-phenyl-1,2- propanedione (V) was also investigated in rat and rabbit. Reduction yielded three metabolites of interest to this study; 1-hydroxy-1-phenyl-2-propanone (VI), 2-hydroxy-1-phenyl-1-propanone (VII) and 1-phenyl-1,2-propanediol (VIII) which was obtained as a mixture of its diastereoisomers (erythro- and threo- VIII) (Table 17). Neither of the intermediate ketol metabolites (VI nor VII) could be completely derivatized with either chiral reagent (MCF or MBIC) and therefore, the optical purities of these metabolites could not be established. Similarly, the four-component mixture of optical isomers of the diol, VIII (1S2R-and 1R2S-erythro; 1S2S- and 1R2R-threo) could not be separated using the chiral reagents, but it was possible to separate the diastereoisomers as their trifluoroacetyl derivatives (Fig. 21).

By applying Prelog's rule, it was possible to predict the stereochemistry of the diol (VIII), assuming that reduction proceeded via the ketoalcohol intermediates (VI, VII) and that the pattern of preferred stereoselectivity remained constant (Fig. 36). Consequently, 1-phenyl-1,2-propanediol

TABLE 17. The Stereochemical Analysis of 1-Phenyl-1,2-propanediol (VIII) obtained from the Metabolic Reduction of 1-Phenyl-1,2-propanedione (V).

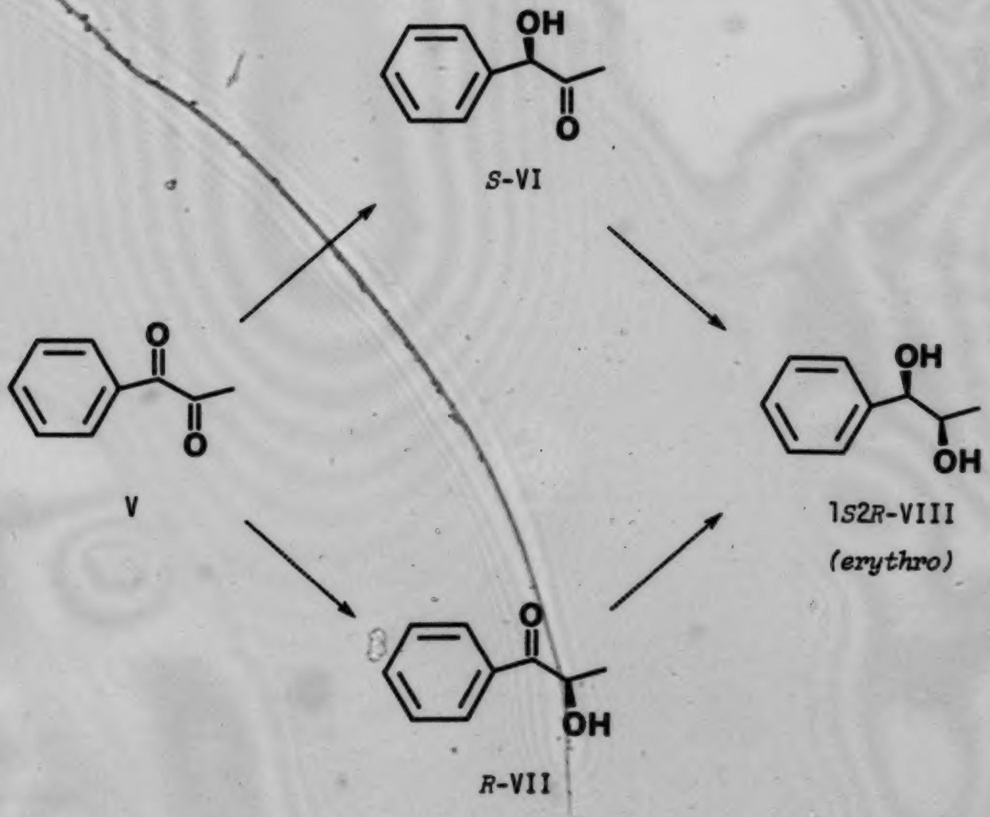
METHODOLOGY			RATIO of DIASTEREOISOMERS of VIII [c]	
SYSTEM	COFACTOR[a]	PRETREATMENT[b]	SPECIES	
			RAT	RABBIT
IN VITRO	NADPH	N/P	97 / 3	97 / 3
	NADH	N/P	94 / 6	98 / 2
IN VIVO	—	N/P	75 / 25	86 / 14
	—	ENZYME	74 / 26	87 / 13
	—	ACID	72 / 28	85 / 15

[a] GENERATING SYSTEM; GLUCOSE-6-PHOSPHATE, $MgCl_2$, and NADP+ or NAD+.

[b] HYDROLYTIC PRETREATMENT OF BIOLOGICAL SAMPLE; N/P -NO PRETREATMENT, ENZYME -β-GLUCURONIDASE (15 000 *U*/ml, INCUBATED 36 Hr. at 37°C), ACID -pH<1 (AUTOCLAVED 40 Min. at 125°C and 15 *psi*.)

[c] RATIO OF DIASTEREOISOMERS, *erythro/threo*-1-phenyl-1,2-propanediol (VIII) as TRIFLUOROACETYLATED DERIVATIVES.

FIGURE 36. Predicted configuration of alcohols obtained from the metabolic reduction of 1-phenyl-1,2-propanedione (V); based on Prelog's rule of preferred product stereoselectivity.


S-VI
V
1S2R-VIII
(erythro)
R-VII

should have occurred predominantly as the 1*R*2*R*-diastereoisomer. Although it was not possible to resolve the 1*R*2*S* and 1*S*2*R* *erythro* isomers, this prediction was accurate inasmuch as the diol (VIII) recovered from the reduction of the diketone *in vitro* by either rat or rabbit liver preparations was mainly (94-98%) in the *erythro* form, the remainder being *threo*-VIII (Table 17). As demonstrated earlier with ketones I and III, the stereoselectivity observed with *in vitro* reduction of 1-phenyl-1,2-propanedione showed minimal variation between rat and rabbit, or between cofactor (NADPH and NADH).

The diol (VIII) recovered from *in vivo* studies was also predominantly the *erythro* diastereoisomer, although a greater amount of the *threo* compound was present compared to what was obtained from *in vitro* studies (Table 16). However, caution should be exercised when attempting to explain this apparent decrease in stereoselectivity, since additional factors such as further metabolism, and differences in distribution and elimination may contribute to the overall variation in recovery of diastereoisomers. There is also the possibility that racemization resulting from the chemical interconversion of the ketol intermediates (VI $\rightleftarrows$ VII) occurred to a small extent (see Section 5.1.1.1.2).

Hydrolysis of urine with either β-glucuronidase or acid caused no change in the ratio of diastereoisomers (VIII) (Table 16). Although this suggested that both the *erythro* and threo isomers were conjugated to the same extent, it was perhaps more likely that due to the highly polar nature of the diols, neither compound (erythro-VIII nor threo-VIII) underwent conjugation prior to being excreted in the urine.

5.2 EXTRAHEPATIC DRUG METABOLISM - THE BIOTRANSFORMATION OF AMPHETAMINE IN RAT BRAIN TISSUE

5.2.1 Introduction

When administered in vivo, the sympathomimetic amine, amphetamine, can undergo a number of metabolic changes depending on the species (175, 512-514). With rat, the predominant metabolite produced and excreted in urine is p-hydroxyamphetamine, along with very small quantities of norephedrine and p-hydroxynorephedrine. In attempts to associate these metabolites with some of the pharmacological actions of amphetamine, their presence in various brain regions and other tissues have been extensively investigated (148, 154, 156-162, 178, 515). But while there is considerable evidence which indicates that tissue levels of these metabolites does influence the intensity of several of the behavioral responses identified with amphetamine, the distribution of these metabolites is unclear. It has been suggested that amphetamine is first para-hydroxylated in the liver (160, 178, 516, 517) and trace amounts of the resulting p-hydroxyamphetamine permeate the blood brain barrier and accumulate in brain tissue where beta-hydroxylation to p-hydroxynorephedrine occurs (170, 179). However, there is alternative evidence from in vitro studies to indicate that brain tissue also has the capability to p-hydroxylate amphetamine (162).

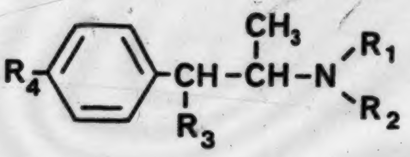
The current investigation was initiated in order to provide information on the extent to which _para-_ and _beta-_ hydroxylation occurs _in vivo_ in cerebral tissue. The disposition of amphetamine, p-hydroxyamphetamine, norephedrine, and _p_-hydroxynorephedrine was studied in brain of rats treated with a single dose (_ip._) of each of these compounds. In addition, parallel _in vitro_ metabolism studies were carried out using NADPH-fortified rat brain 10 000X_g_ supernatant.

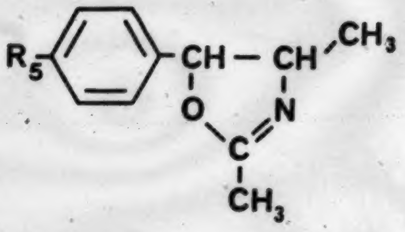
5.2.2 Isolation, Identification and Quantitation of Trace Metabolites from Biological Samples

Amphetamine (XII), norephedrine (XIV), and their metabolites, _p_-hydroxyamphetamine (XIII) and _p_-hydroxynorephedrine (XV) were expected to be present in brain samples in trace concentrations. Various methods have been developed for the determination of trace quantities of these and related amines in aqueous samples. Procedures are generally complex and employ tritiated compounds (148, 151, 154, 158, 162, 179, 515, 516) or the extraction of the compounds into an organic solvent followed by derivatization with a reagent sensitive to electron-capture detection (163, 178, 181). This latter extraction-derivatization procedure was confirmed to be successful for the analysis of several amines if present in sufficient quantities, but a poor sensitivity was demonstrated for phenolic amines which were normally inefficiently extracted from aqueous solution. In addition, a major problem involving extractions of biological samples with organic solvents was the co-extraction of endogenous compounds which subsequently interfered in the GLC analyses. This not only hampered attempts to analyze compounds in the sub-microgram range, but also resulted in a poor reproducibility at lower concentrations. For the brain analysis in the current study, an extraction procedure developed by Coutts, Baker, and coworkers (518-520) was adapted which permitted quantitiative recoveries of all metabolites, as their acetylated derivatives, from biolgical samples. Subsequent perfluoroacylating reactions with PFPA or TFAA and the use of EC-GLC successfully permitted the assay of amines XII-XV to low ng levels.

While investigating the GLC properties of biogenic amines as their acylated and/or perfluoroacylated derivatives, it was necessary to find the optimum parameters for the separation of these compounds. Different column liquid phases with varied polarities were tested, and the silicones, OV-17 and OV-101, were found most suitable. Shown in Table 18 are the retention times of the parent amines and their derivatives when chromatographed on 5% OV-101. Identification of the GLC peaks was achieved by mass spectral analysis.

TABLE 18. Structures and GLC Retention Times of the Acetylated and/or Perfluoroacylated Derivatives of the Biogenic Amines Tested: Amphetamine (XII); *p*-Hydroxyamphetamine (XIII); Norephedrine (XIV); *p*-Hydroxynorephedrine (XV).





COMPOUND	R_1	R_2	R_3	R_4	R_5	RETENTION TIME (min)[1]
XII	H	H	H	H		1.20
XIII	H	H	H	OH		8.30
XIV	H	H	OH	H		3.45
XV	H	H	OH	OH		∞[2]
XIIa	H	$COCH_3$	H	H		7.35
XIIIa	H	$COCH_3$	H	$OCOCH_3$		11.0[3]
XIVa	H	$COCH_3$	OH	H		8.0[3]
XVa	H	$COCH_3$	OH	$OCOCH_3$		15.0[3]
XIIb	COC_2F_5	$COCH_3$	H	H		3.00
XIIIb	COC_2F_5	$COCH_3$	H	$OCOCH_3$		15.0[3]
XIVb	COC_2F_5	$COCH_3$	$OCOC_2F_5$	H		1.60
XVb	COC_2F_5	$COCH_3$	$OCOC_2F_5$	$OCOCH_3$		5.0[3]
XIVc	--	--	--	--	H	3.10
XVc	--	--	--	--	$OCOCH_3$	2.0[4]

TABLE 18 continued.

XIIId	H	$COCH_3$	H	OH		19.0[3]
XVd	H	$COCH_3$	OH	OH		∞[2]
XIIIe	COC_2F_5	$COCH_3$	H	$OCOC_2F_5$		3.75
XVe	COC_2F_5	$COCH_3$	$OCOC_2F_5$	$OCOC_2F_5$		1.65
XIIIf	H	$COCH_3$	H	$OCOC_2F_5$		8.80
XVf	--	--	--	--	$OCOC_2F_5$	4.05
XIIg	COC_2F_5	H	H	H		1.95
XIIIg	COC_2F_5	H	H	$OCOC_2F_5$		3.00
XIVg	COC_2F_5	H	$OCOC_2F_5$	H		1.55
XVg	COC_2F_5	H	$OCOC_2F_5$	$OCOC_2F_5$		2.00
XIIIh	H	$COCH_3$	H	$OCOCF_3$		9.45
XVh	--	--	--	--	$OCOCF_3$	4.20
XIIi	$COCF_3$	H	H	H		2.35
XIIIi	$COCF_3$	H	H	$OCOCF_3$		3.90
XIVi	$COCF_3$	H	$OCOCF_3$	H		1.90
XVi	$COCF_3$	H	$OCOCF_3$	$OCOCF_3$		2.85

---

[1] RETENTION TIME: 5% OV-101 (1.26 m; 4 mm i.d.), OVEN TEMPERATURE PROGRAMMING, 120^{0} (4 min) $\longrightarrow$ 180^{0} @ 4^{0}/min.

[2] NOT ELUTED BY GLC AT ANY TEMPERATURE.

[3] OVEN TEMPERATURE 180^{0} (ISOTHERMAL).

[4] OVEN TEMPERATURE 250^{0} (ISOTHERMAL).

5.2.2.1 Derivatizations

5.2.2.1.1 Acetylation/Extraction

Efficient extraction of the amphoteric metabolites (XII, XV) into an organic solvent was made possible by their prior aqueous acetylation to lipophilic N,O-diacetates (XIIa, XV[illegible] (Fig. 37A). Acetic anhydride (AA) was used successfully for the preparation of these derivatives. The reaction proceeded rapidly at room temperature using AA in an amount equal to one-tenth the volume of the aqueous sample. This acetylation procedure also converted amphetamine (XII) and norephedrine (XIV) into their N-acetates (XIIa and XIVa, respectively) (Fig. 37B). The β-hydroxyl group of both XIV or XV did not undergo acetylation. In this one-step derivatization and extraction method the acetylated amines, exhibiting a more lipophilic nature, were extracted from the aqueous phase at neutral pH into the organic layer (ethyl acetate) in significantly improved yields. The recovered acetates (XIIa - XVa) all demonstrated an excellent thermal stability which permitted GLC analysis at very high temperatures (>250°C).

In Table 19 are summarized the diagnostic fragmentation ions of the parent and acetylated amines. All mono- and diacetates (XIIa, XIIIa, XIVa, XVa, XIIId, XVd) gave mass spectra which could be characterized by the production of $CH_3CH = NHCOCH_3^+$ (m/z 86), $CH_3CH = NH_2^+$ (m/z 44) and $CH_3C\equiv O^+$ (m/z 43) fragments. The structures of the

FIGURE 37. Derivatization scheme for the acylation of:
A) *p*-hydroxyamphetamine (XIII) and *p*-hydroxynorephedrine (XV);
B) amphetamine (XII) and norephedrine (XIV).
(AA = Acetic Anhydride)

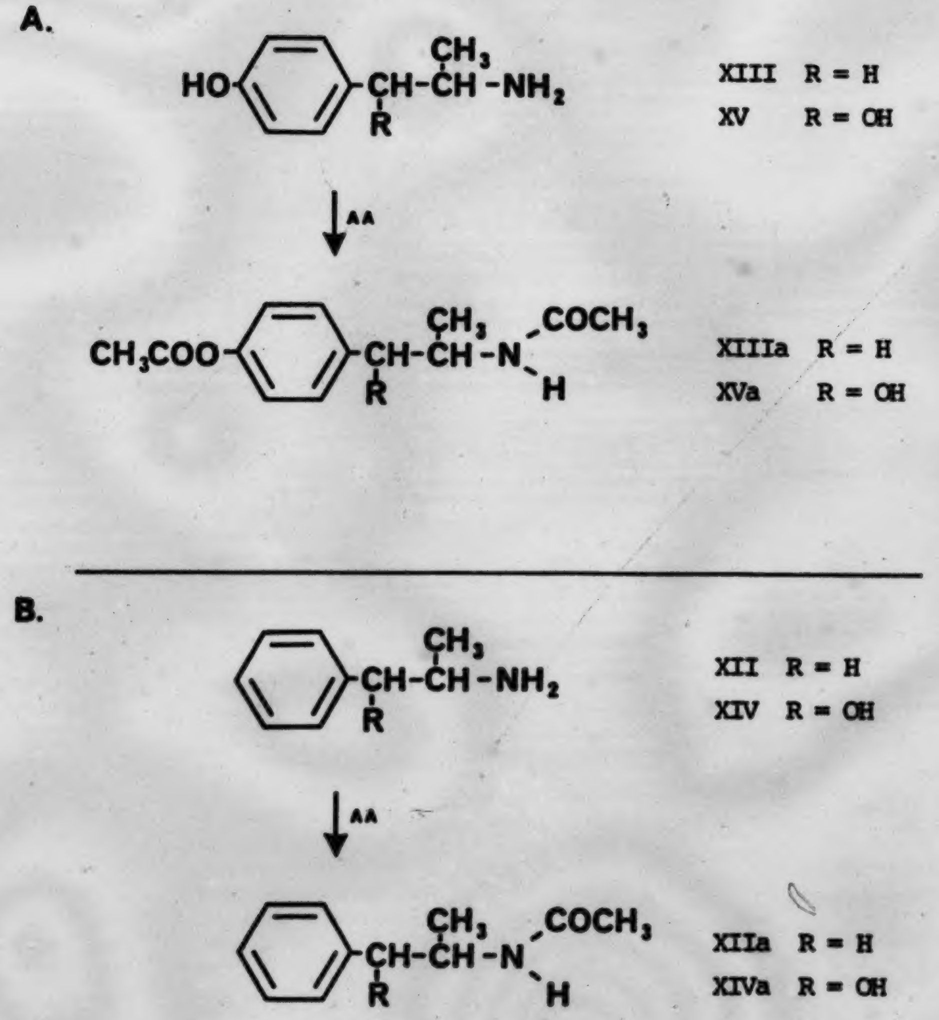

A.
HO
CH₃
CH-CH-NH₂
R
XIII R = H
XV R = OH
AA
CH₃COO
CH₃
CH-CH-N
COCH₃
H
R
XIIIa R = H
XVa R = OH
B.
CH₃
CH-CH-NH₂
R
XII R = H
XIV R = OH
AA
CH₃
CH-CH-N
COCH₃
H
R
XIIa R = H
XIVa R = OH

TABLE 19. EI-Mass Spectral Data of the Parent Biogenic Amines (XII-XV) and Their Acetylated Derivatives.

COMPOUND	m/z (% RELATIVE ABUNDANCE)
XII	135 (absent)[$M^{+\cdot}$]; 120(4)[**Ph**-$CH_2CH{=}NH_2^+$]; 91(16) [$C_7H_7^+$]; 65(10)[$C_5H_5^+$]; 44(100)[$CH_3CH{=}NH_2^+$].
XIII	151(5)[$M^{+\cdot}$]; 107(26)[HO-C_6H_4-CH_2^+]; 91(5); 79(6) [(m/z 107 – CO)$^+$]; 77(24)[$C_6H_5^+$]; 44(100). *
XIV	151 (absent)[$M^{+\cdot}$]; 107(5)[**Ph**-$CHOH^+$]; 105(7)[**Ph**-$C{\equiv}O^+$]; 91(3); 79(11)[(m/z 107 – CO)$^+$]; 77(23); 44(100). *
XV	167 (absent)[$M^{+\cdot}$]; 123(6)[HO-C_6H_4-$CHOH^+$]; 95(5) [(m/z 123 – CO)$^+$]; 77(4); 44(100). *
XIIa[1]	177(6)[$M^{+\cdot}$]; 118(100)[**Ph**-$CH{=}CHCH_3^{+\cdot}$]; 117(47) [(m/z 118 – ·H)$^+$]; 91(75); 86(82)[$CH_3CH{=}NHCOCH_3^+$]; 65(23); 44(52); 43(19)[$CH_3C{\equiv}O^+$]. *
XIIIa[1]	235 (absent)[$M^{+\cdot}$]; 176(81)[(M – $CH_3CONH_2^{+\cdot}$]; 134(100) [HO-C_6H_4-$CH{=}CHCH_3^{+\cdot}$]; 133(24)[(m/z 134 - ·H)$^+$]; 107(57); 105(7); 86(65); 79(8); 77(19); 44(61); 43(23). *
XIVa[2]	193 (absent)[$M^{+\cdot}$]; 107(11); 105(11); 87(100)[$C_4H_9ON^{+\cdot}$]; 86(43)[$CH_3CH{=}NHCOCH_3^+$]; 79(17); 77(23); 72(21) [(m/z 87 – ·CH_3)$^+$]; 44(38); 43(11). *
XVa[3]	251 (absent)[$M^{+\cdot}$]; 233(11)[(m/z 251 – H_2O)$^{+\cdot}$]; 191(47) [(m/z 233 – $CH_2{=}C{=}O$)$^+$]; 149(100)[HO-C_6H_4-$CH{=}C(CH_3)NH_2^{+\cdot}$]; 134(18)[(m/z 149 – ·$CH_3$)$^+$]; 107(17); 105(5); 79(6); 77(15); 43(26). *

TABLE 19 continued.

XIIId[1]	193 (absent)[$M^{+\cdot}$]; 134(100)[$(M - CH_3CONH_2)^{+\cdot}$]; 133(25) [$(m/z\ 134 - H)^{+}$]; 107(40); 86(31); 77(25); 44(59); 43(12). *
XVd[2]	209 (absent)[$M^{+\cdot}$]; 150(29)[$(M - CH_3CONH_2)^{+\cdot}$]; 123(20); 121(21)[$(m/z\ 123 - H_2)^{+}$]; 87(100); 77(31); 72(38); 44(60); 43(41). *

---

[1] FRAGMENTATION PROFILE SHOWN IN FIGURE 38.

[2] FRAGMENTATION PROFILE SHOWN IN FIGURE 39.

[3] FRAGMENTATION PROFILE SHOWN IN FIGURE 40.

* THE IDENTITIES OF IONS NOT LABELLED ARE GIVEN ELSEWHERE IN THE TABLE.

amphetamine N-monoacetates (XIIa, XIIId) were further confirmed by the presence of major ions at m/z M-59 [(M- NH_2COCH_3)+] and M-60 [$(M-59-H)^+$] (Fig. 38). The N, O-diacetate derivative (XIIIa) of p-hydroxyamphetamine had a fragmentation profile very similar to that of its N-monoacetate analog (XIIId), although its O-acetate moiety accounted for the one extra ion at m/z 176 (Fig. 38).

Characteristic ions in the spectra of the acetylated alcoholamines are illustrated in Fig. 39 for the N-monoacetates (XIVa, XVd) and Fig. 40 for the N, O-diacetate derivative (XVa). N-Acetylnorephedrine (XIVa) and N-acetyl-p-hydroxynorephedrine (XVd) both gave a particularly diagnostic fragment (m/z 87) as their base peaks, which resulted from the loss of the benzaldehyde moiety from the molecular ion. The ensuing expulsion of a methyl radical formed the m/z 72 fragment. N-Acetylnorephedrine (XIVa) further showed a fragment arising from β-cleavage of its benzalcohol group (m/z 86) which, although characteristic for all the N-acetyl derivatives of the amphetamines (XIIa, XIIIa, XIIId), was not observed in alcoholamine derivatives other than XIVa. With N, O-diacetyl-p-hydroxynorephedrine (XVa), it was interesting to note that the spectrum was consistent with its dehydrated product (Fig. 41). A similar effect was observed by Coutts et al. (521) with the N,O-diacetate derivatives of p-octopamine and normetanephrine. Apparently, dehydration occurred either on the GLC column or in the mass

FIGURE 38. EI-MS fragmentation profiles of N-acetylamphetamine (XIIa), N-acetyl-*p*-hydroxyamphetamine (XIIId), and N,O-diacetyl-*p*-hydroxyamphetamine (XIIIa).

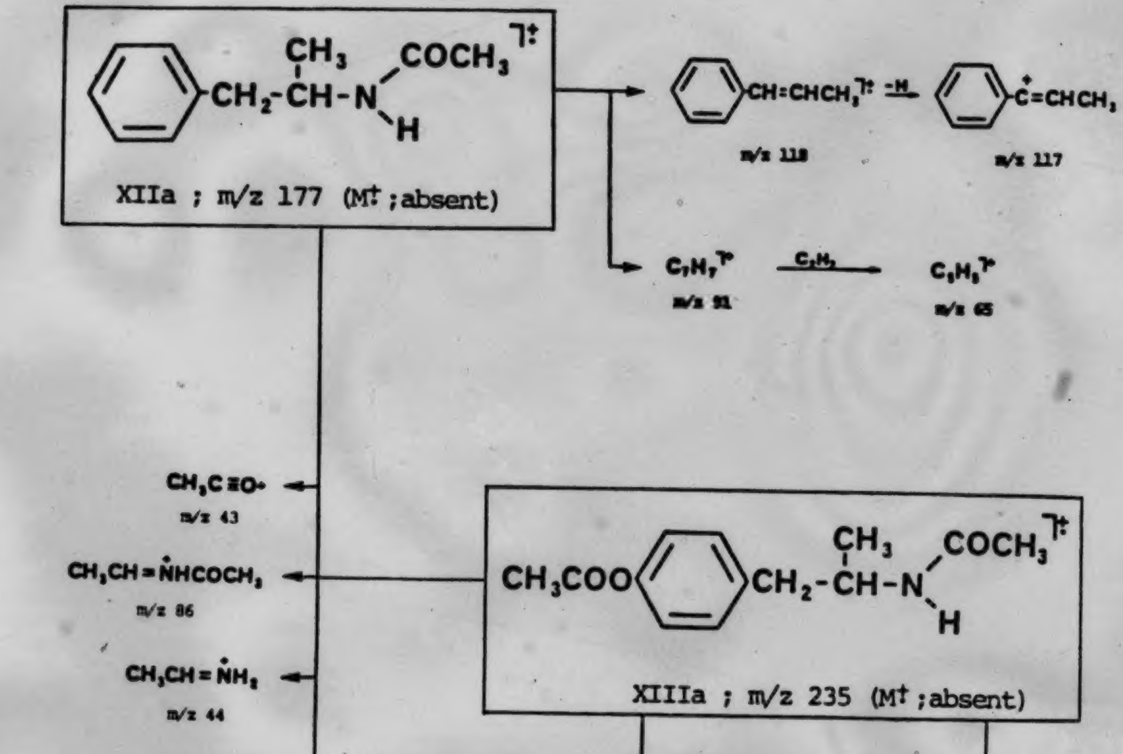

XIIa ; m/z 177 (M⁺ ; absent)
-H
m/z 118
m/z 117
C7H7
C2H2
C5H5
m/z 91
m/z 65
CH3C≡O+
m/z 43
CH3CH=NHCOCH3
m/z 86
CH3CH=NH2
m/z 44
XIIIa ; m/z 235 (M⁺ ; absent)
m/z 107
m/z 176
-CO
-CH2=C=O
C6H7
m/z 79
m/z 134
-H2
-H
C6H5
m/z 77
m/z 133
XIIId ; m/z 193 (M⁺ ; absent)

FIGURE 39. EI-MS fragmentation profiles of N-acetylnorephedrine (XIVa) and N-acetyl-*p*-hydroxynorephedrine (XVd).

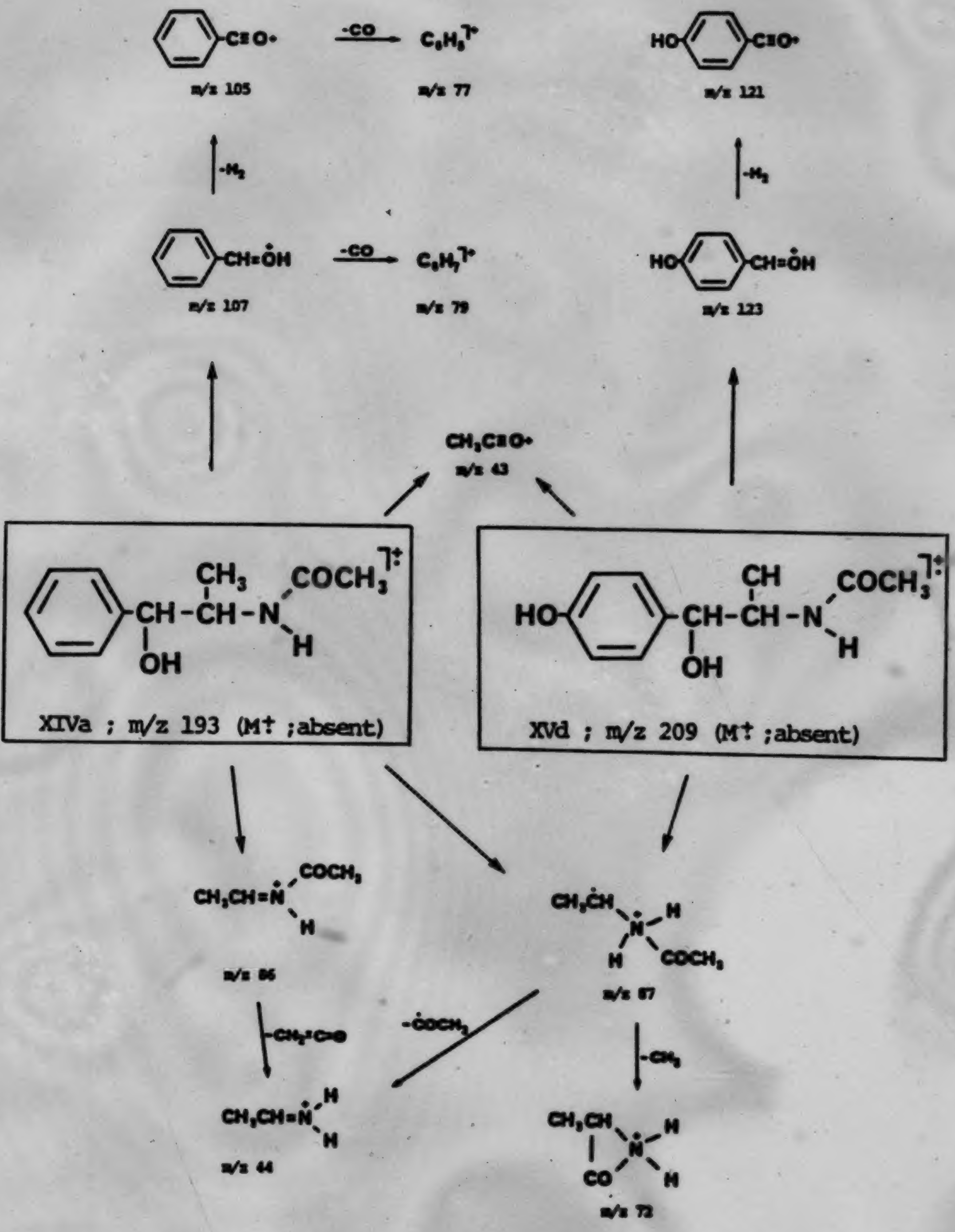

m/z 105
-CO
C_6H_5
m/z 77
m/z 121
$-H_2$
$-H_2$
m/z 107
-CO
C_6H_7
m/z 79
m/z 123
$CH_3C\equiv O$
m/z 43
XIVa ; m/z 193 (M+ ;absent)
XVd ; m/z 209 (M+ ;absent)
m/z 86
m/z 87
$-CH_2=C=O$
$-\dot{C}OCH_3$
$-CH_3$
m/z 44
m/z 72

FIGURE 40. EI-MS fragmentation profile of N,O-diacetyl-*p*-hydroxy-norephedrine (XVa).

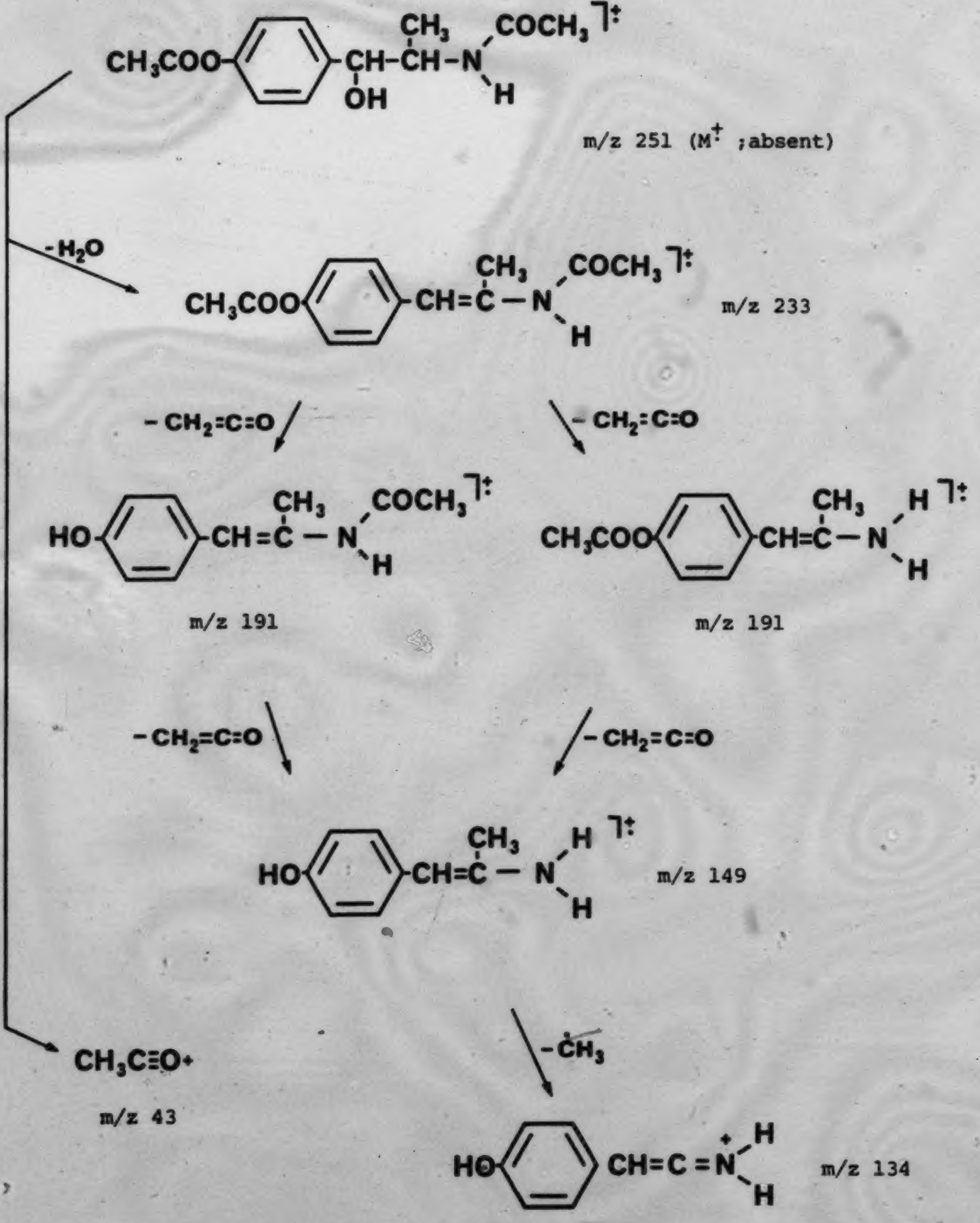
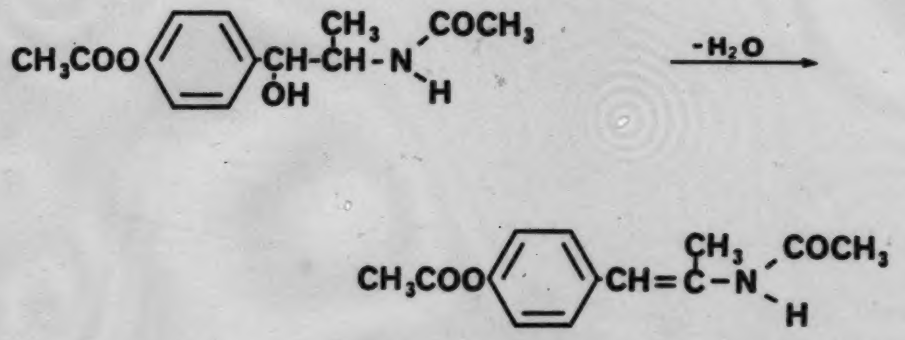

m/z 251 (M$^{+\cdot}$;absent)
-H$_2$O
m/z 233
- CH$_2$=C=O
- CH$_2$=C=O
m/z 191
m/z 191
- CH$_2$=C=O
- CH$_2$=C=O
m/z 149
-$\dot{C}$H$_3$
CH$_3$C≡O+
m/z 43
m/z 134

FIGURE 41. Reaction scheme for the dehydration of N,O-diacetyl-*p*-hydroxynorephedrine (XVa).

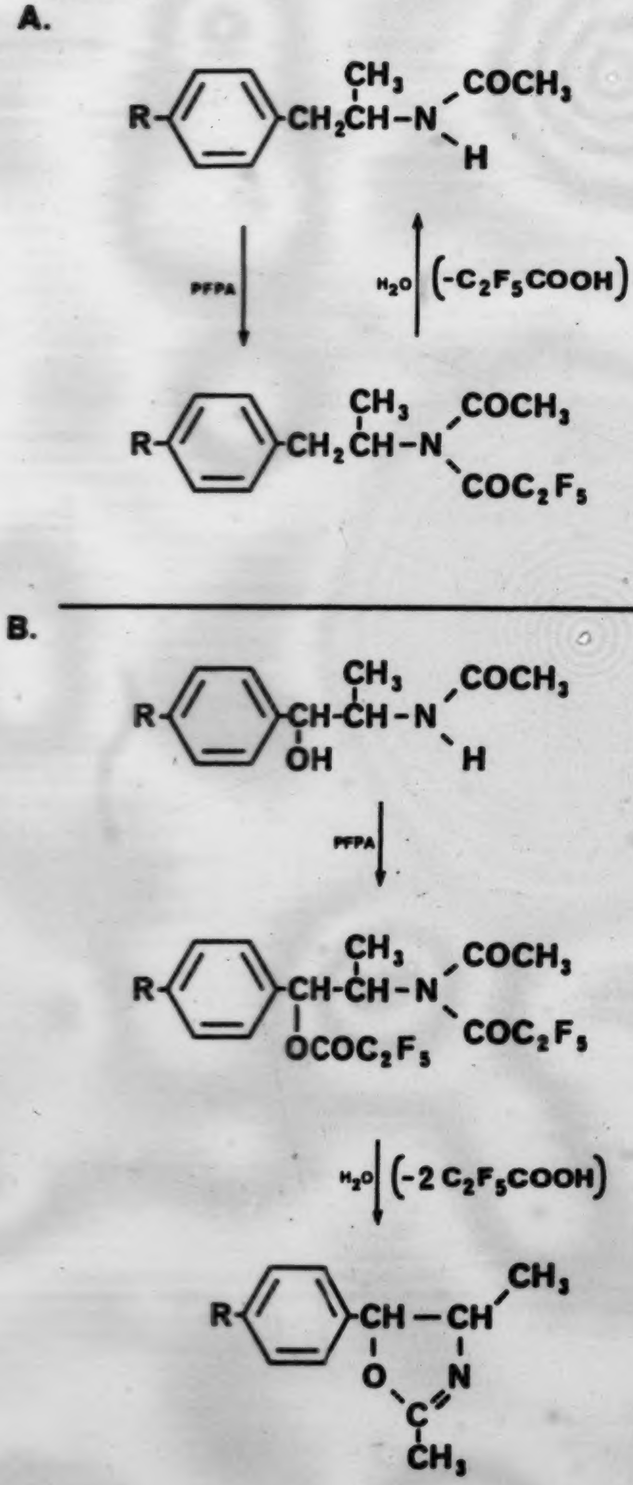


spectrometer, in view of the fact that perfluoroacylation of the β-hydroxyl group of the diacetate was still possible (Fig. 42B). In contrast, the N-acetyl derivative of either norephedrine (XIVa) or p-hydroxynorephedrine (XVd) did not appear to dehydrate. Exactly why the presence of a phenolic acetate moiety seemed to promote the loss of H_2O from phenylalcoholamines is unclear.

5.2.2.1.2 Pentafluoropropionylation

Acetate derivatives unfortunately, possessed poor chromatographic properties, including extremely long GLC retention times (Table 18) and low sensitivity to flame ionization detection. Injected samples of less than 1 ug/ml concentration were usually lost in the baseline. Attempts were made to pentafluoropropionylate the extracted acetates (XIIa - XVa) to increase their volatility (522-524) and improve their detection capabilities by making them sensitive to electron - capture detection (EC) (Fig. 42). Mass spectral data of the resulting pentafluoropropionylated compounds (XIIb-XVb) are presented in Table 20. It was found however, that N-isopropyl , N-acetate biogenic amines were not eluted as single peaks when chromatographed as the perfluoroacylated derivatives. This indicated that either the perfluoroacylating reaction was incomplete, or that the resulting derivatives were unstable. The analyses of solutions of XIIb, XIIIb, XIVb, or XVb at regular time intervals by GLC-MS and EC-GLC, revealed that all four derivatives degraded into products which were

FIGURE 42. Derivatization scheme for the pentafluoropropionylation of the acetylated amines: A) *N*-acetylamphetamine (XIIa) and *N*,*O*-diacetyl-*p*-hydroxyamphetamine (XIIIa); B) *N*-acetylnorephedrine (XIVa) and *N*,*O*-diacetyl-*p*-hydroxynorephedrine (XVa). (PFPA = pentafluoropropionic anhydride)



A.
CH₃
COCH₃
R
CH₂CH–N
H
PFPA
H₂O
(-C₂F₅COOH)
COC₂F₅
B.
CH-CH-N
OH
OCOC₂F₅
(-2 C₂F₅COOH)
CH–CH
O
N
C

XIIa R = H

XIIIa R = CH_3COO

XIIb R = H

XIIIb R = CH_3COO

XIVa R = H

XVa R = CH_3COO

XIVb R = H

XVb R = CH_3COO

XIVc R = H

XVc R = CH_3COO

TABLE 20. EI Mass Spectral Data of the Pentafluoropropionyl Derivatives of Acetylated Amines.

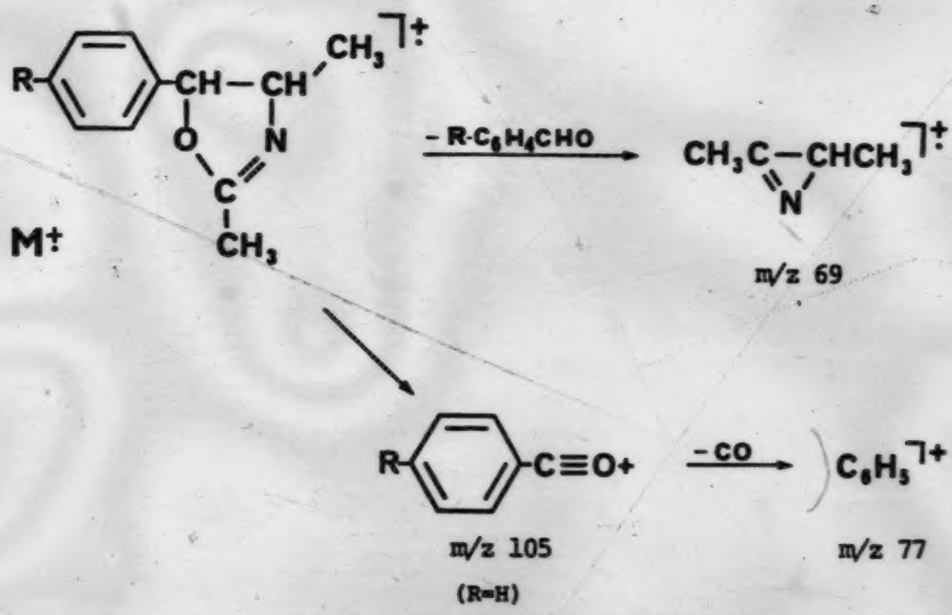
COMPOUND	m/z (% RELATIVE ABUNDANCE)
XIIb	323 (absent)[$M^{+\cdot}$]; 190(17)[$CH_3CH=NHCOC_2F_5^+$]; 119(36)[$C_2F_5^+$]; 118(83)[$Ph\text{-}CH=CHCH_3^{+\cdot}$]; 117(29)[$Ph\text{-}C=CHCH_3^+$]; 91(100)[$C_7H_7^+$]; 69(27)[$CF_3^+$]; 65(22)[$C_5H_5^+$]; 43(35)[$CH_3C{\equiv}O^+$].
XIIIb	381 (absent)[$M^{+\cdot}$]; 190(22); 176(16)[$CH_3COO\text{-}C_6H_4\text{-}CH=CHCH_3^{+\cdot}$]; 135(18)[$HO\text{-}C_6H_4\text{-}CH_2CHCH_3^+$]; 134(67)[$HO\text{-}C_6H_4\text{-}CH=CHCH_3^{+\cdot}$]; 119(19); 107(100)[$HO\text{-}C_6H_4\text{-}CH_2^+$]; 43(20). *
XIVb	485 (absent)[$M^{+\cdot}$]; 232(10)[$CH_3CH=N(COCH_3)COC_2F_5^+$]; 190(35); 119(36); 105(20); 77(19)[$C_6H_5^+$]; 69(36); 43(100). *
XVb	543 (absent)[$M^{+\cdot}$]; 296(27)[$HO\text{-}C_6H_4\text{-}CH(OCOC_2F_5)CH=CH_2^{+\cdot}$]; 190(75); 169(100)[$HO\text{-}C_6H_4\text{-}CH=OCOC_2F_5^+$]; 119(51); 77(12); 69(16); 43(26). *

* THE IDENTITIES OF IONS NOT LABELLED ARE GIVEN ELSEWHERE IN THE TABLE.

no longer sensitive to EC detection. The derivatives of amphetamine (XIIb) and p-hydroxyamphetamine (XIIIb) rapidly lost the N-pentafluoropropionyl moiety by hydrolysis (Fig. 42A; XIIa and XIIIa, respectively), whereas the derivatives of norephedrine (XIVb) and p-hydroxynorephedrine (XVb) slowly cyclized to EC-insensitive oxazoline products (Fig. 42B; XIVc and XVc, respectively). The structure of the oxazoline derivatives were deduced from their mass spectral behavior (Table 21). Plausible mechanisms for the formation of the oxazolines (XIVc, XVc, XVf, XVh) as proposed by Coutts et al. (521) are illustrated in Figure 43.

The problem of insufficient EC-GLC sensitivity was overcome for p-hydroxyamphetamine and p-hydroxynorephedrine by the preferential hydrolysis of the O-acetate moiety of their N,O-diacetates by means of ammonium hydroxide (Fig. 44). This treatment freed the phenolic OH group for reaction with the perfluoroacylating reagent, producing compounds XIIId and XVd. As before, the N-pentafluoropropionyl moiety rapidly hydrolyzed from the p-hydroxyamphetamine derivative (XIIId) to form XIIIf, and the p-hydroxynorephedrine derivative (XVd) cyclized to produce the oxazoline, XVf. However, these products remained sensitive to electron capture detection due to the presence of the O-pentafluoropropionyl group, which was stable to hydrolysis for at least two weeks when the derivative was refrigerated. Furthermore, the resulting derivatives (XIIIf, XVf) had

TABLE 21. Diagnostic EI Fragment Ions and Fragmentation Pattern of the Oxazoline Derivatives.

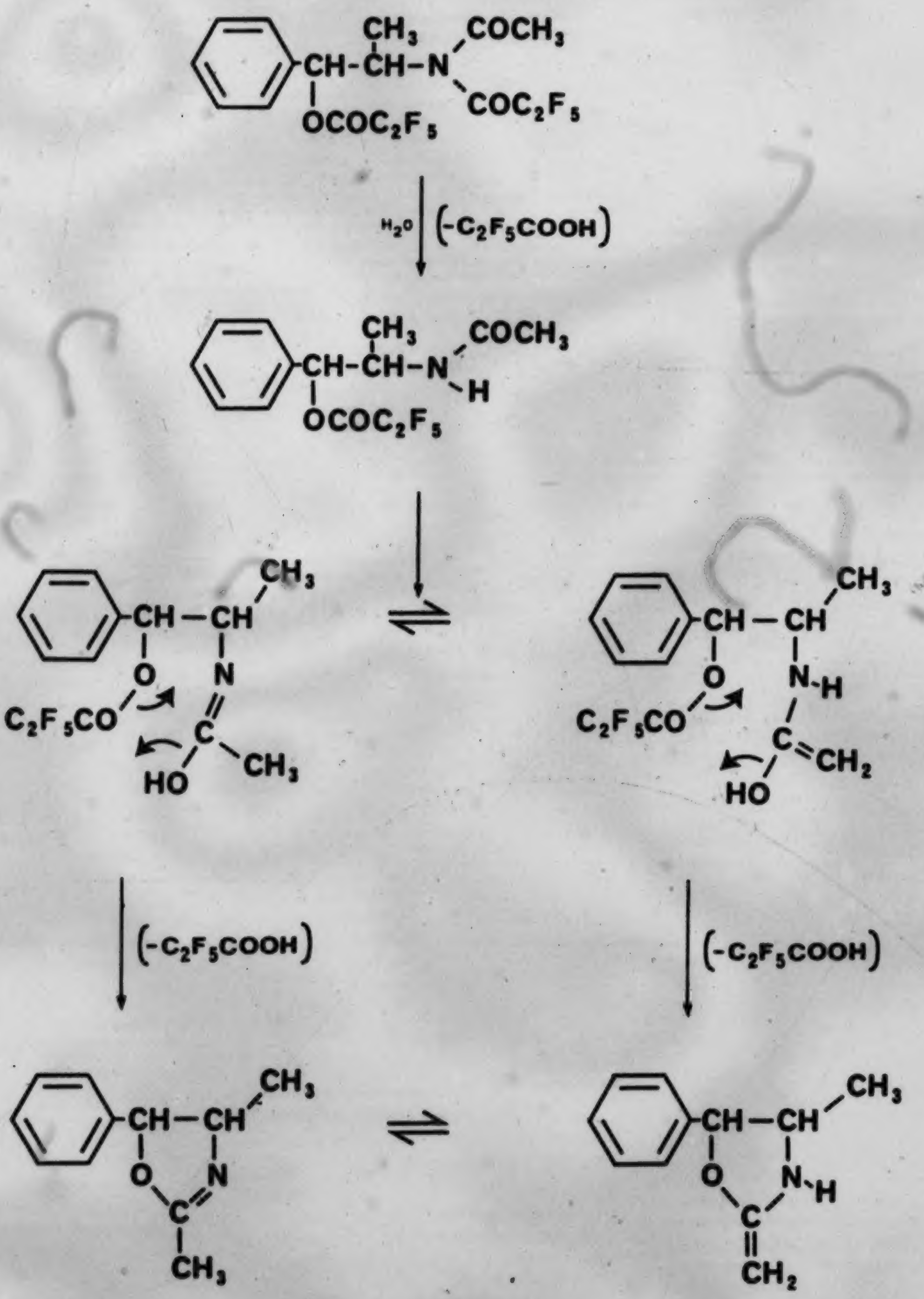


M$^{+\cdot}$
$-\mathrm{R\text{-}C_6H_4CHO}$
m/z 69
$-\mathrm{CO}$
m/z 105
(R=H)
m/z 77

COMPOUND	R	m/z (% RELATIVE ABUNDANCE)
XIVc	H	175(6)[M$^{+\cdot}$]; 105(8); 77(10); 69(100).
XVc	$OCOCH_3$	233(6)[M$^{+\cdot}$]; 190(4)[(M - CH_2=C=O)$^{+\cdot}$]; 119(14) [$C_2F_5^+$]; 69(100).
XVf	$OCOC_2F_5$	337 (absent)[M$^{+\cdot}$]; 190(4)[(M - COC_2F_5)$^+$]; 119(14)[$C_2F_5^+$]; 69(100).
XVh	$OCOCF_3$	287 (absent)[M$^{+\cdot}$]; 69(100)[1].

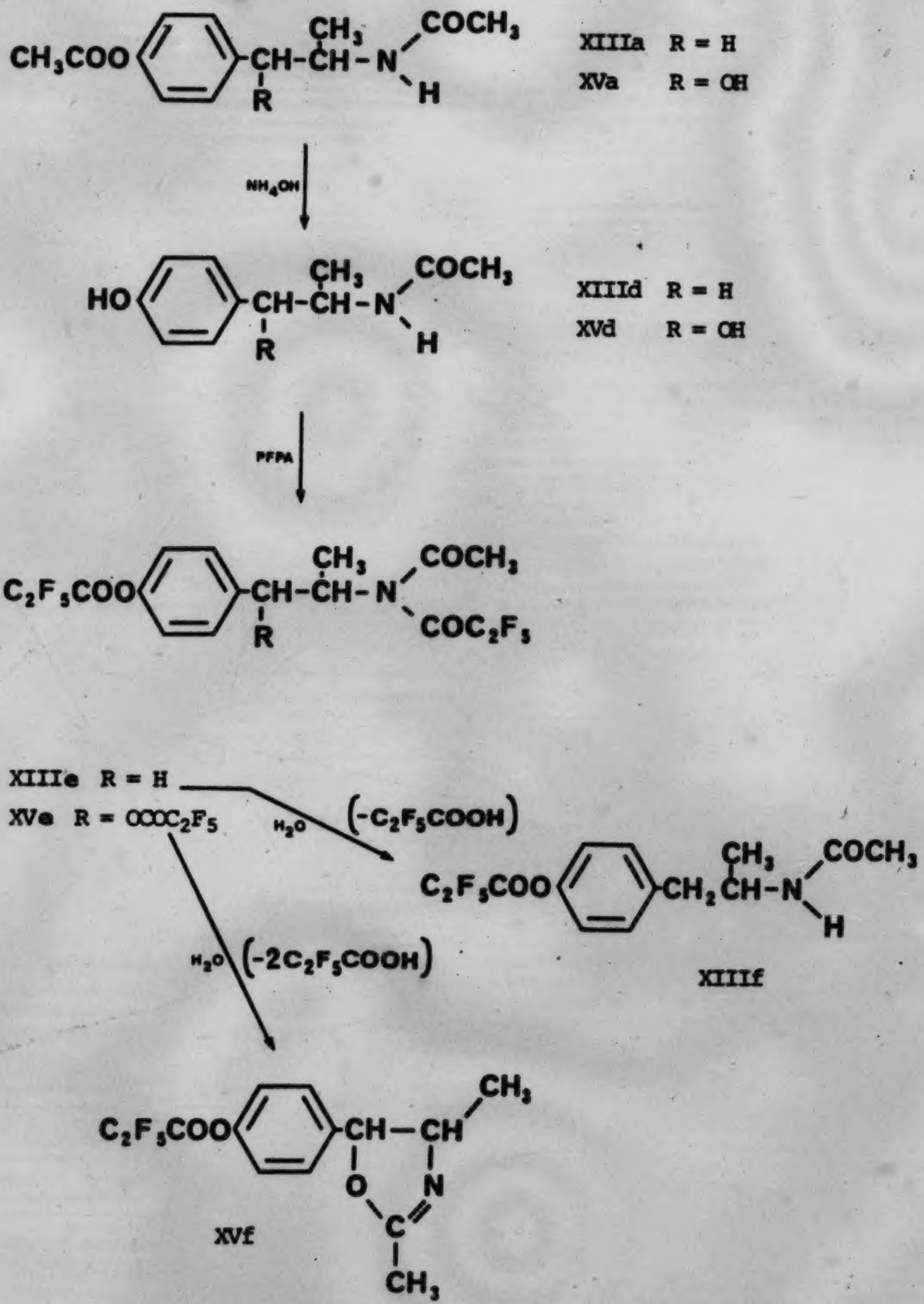
[1] ALSO INCLUDES A MINOR CONTRIBUTION BY CF_3^+

FIGURE 43. Plausible mechanisms for the formation of the oxazoline structure. [As proposed by Coutts *et al.* (521)]



CH₃
COCH₃
CH-CH-N
COC₂F₅
OCOC₂F₅
H₂O
(-C₂F₅COOH)
CH₃
COCH₃
CH-CH-N
H
OCOC₂F₅
CH₃
CH-CH
O
N
C₂F₅CO
C
HO
CH₃
CH₃
CH-CH
O
N-H
C₂F₅CO
C
CH₂
HO
(-C₂F₅COOH)
(-C₂F₅COOH)
CH₃
CH-CH
O
N
C
CH₃
CH₃
CH-CH
O
N-H
C
CH₂

FIGURE 44. Derivatization scheme of the pentafluoropropionylation of the N,O-diacetates of *p*-hydroxyamphetamine (XIIIa) and *p*-hydroxynorephedrine (XVa) following prior preferential hydrolysis of their O-acetate moiety with ammonium hydroxide. (PFPA = pentafluoropropionic anhydride).


XIIIa R = H
XVa R = OH
NH₄OH
XIIId R = H
XVd R = OH
PFPA
XIIIe R = H
XVe R = OCOC₂F₅
H₂O
(-C₂F₅COOH)
XIIIf
H₂O
(-2C₂F₅COOH)
XVf

satisfactory properties for both gas chromatography and mass spectrometry. The structures of the pentafluoropropionylated products illustrated in Figure 44 were confirmed by EI-MS; the major fragments are given in Table 22. Suggested fragmentation pathways for the p-hydroxyamphetamine derivative (XIIIf) are given in Figure 45. The general fragmentation pattern of oxazolines, including XVf, is included in Table 21.

Since neither amphetamine (XII) nor norephedrine (XIV) contain a phenolic group, the analytical procedure just described for the analysis of p-hydroxyamphetamine (XIII) and p-hydroxynorephedrine (XV) was not applicable to the quantitation of XII and XIV. Therefore, a separate analytical procedure was necessary for the latter two amines. Following the ion exchange step in the extraction procedure (Section 4.4.2.1.2), XII and XIV were directly removed from the dilute hydrochloric acid solution referred to above which contained the four amines (XII-XV) by basifying the solution (pH > 9) and extracting it with ethyl acetate. The extracts, containing XII and XIV, were reacted with PFPA, giving N-pentafluoropropionyl derivatives (Fig. 46) which were EC-GLC sensitive, and did not hydrolyze in the presence of water. This procedure permitted effective recovery and detection of both amphetamine (XII) and norephedrine (XIV) without interference from either of the amphoteric compounds (XIII, XV), or from endogenous components. PFPA

TABLE 22. EI Mass Spectral Data of the O-Pentafluoropropionyl Derivatives of the N-Acetyl Phenolic Amines (XIIIf, XVf), and of Their Unstable Precursors (XIIIe, XVe).

COMPOUND	m/z (% RELATIVE ABUNDANCE)
XIIIe	485 (absent)[$M^{+\cdot}$]; 281(33)[$C_2F_5COO\text{-}C_6H_4\text{-}CH_2CHCH_3^+$]; 280(100)[$C_2F_5COO\text{-}C_6H_4\text{-}CH{=}CHCH_3^{+\cdot}$]; 253(64)[$C_2F_5COO\text{-}C_6H_4\text{-}CH_2^+$]; 225(14)[(m/z 253 - CO)$^+$]; 190(29)[$CH_3CH{=}NHCOC_2F_5^+$]; 119(66)[$C_2F_5^+$]; 69(23)[CF_3^+]; 43(64)[$CH_3C{\equiv}O^+$].
XVe	647 (absent)[$M^{+\cdot}$]; 232(10)[$CH_3CH{=}N(COCH_3)COC_2F_5^+$]; 190(41)[(m/z 232 - CH=C=O)$^+$]; 140(14)[unknown]; 119(43); 69(33); 43(100). *
XIIIf[1]	339 (absent)[$M^{+\cdot}$]; 280(20); 253(7); 225(3); 119(27); 86(100)[$CH_3CH{=}NHCOCH_3^+$]; 44(83)[$CH_3CH{=}NH_2^+$]; 43(20). *
XVf[2]	337 (absent)[$M^{+\cdot}$]; 190(4)[$(M - COC_2F_5)^+$]; 119(14); 69(100). *

[1] FRAGMENTATION PROFILE SHOWN IN FIGURE 44.

[2] FRAGMENTATION PROFILE SHOWN IN TABLE 21.

* THE IDENTITIES OF IONS NOT LABELLED ARE GIVEN ELSEWHERE IN THE TABLE.

FIGURE 45. EI-MS fragmentation profile of N-acetyl,O-pentafluoropropionyl-*p*-hydroxyamphetamine (XIIIf).

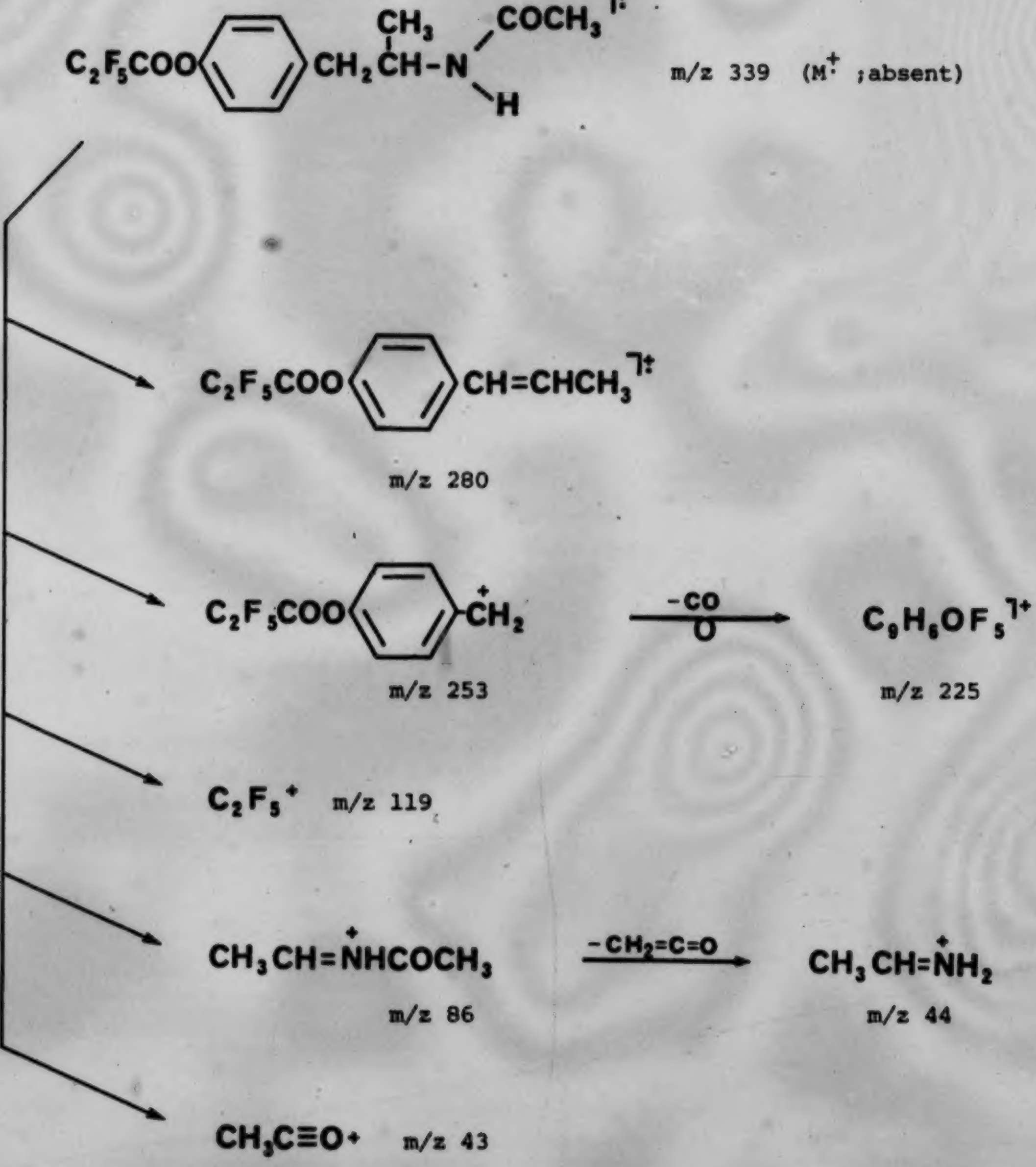
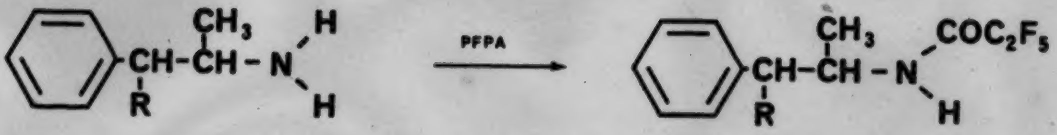

C2F5COO CH2CH(CH3)-N(COCH3)H]+·
m/z 339 (M+· ;absent)
C2F5COO CH=CHCH3]+·
m/z 280
C2F5COO CH2+
m/z 253
-CO
O
C9H6OF5]+
m/z 225
C2F5+ m/z 119
CH3CH=N+HCOCH3
m/z 86
-CH2=C=O
CH3CH=N+H2
m/z 44
CH3C≡O+ m/z 43

FIGURE 46. Derivatization scheme of the pentafluoropropionylation of of amphetamine (XII) and norephedrine (XIV). (PFPA = pentafluoropropionic anhydride).



XII R = H

XIV R = OH

XIIg R = H

XIVg R = $OCOC_2F_5$

reactions appeared quantitative as no underivatized products were detected. The resulting derivatives exhibited excellent chromatographic properties. Overall recoveries for the compounds, using the methods described above, were as follows: XII (87.7 ± 0.6%), XIII (65.0 ± 2.7%), XIV (80.3 ± 1.2%) and XV (52.7 ± 2.5%), where values represent mean ± S.D. (n=3).

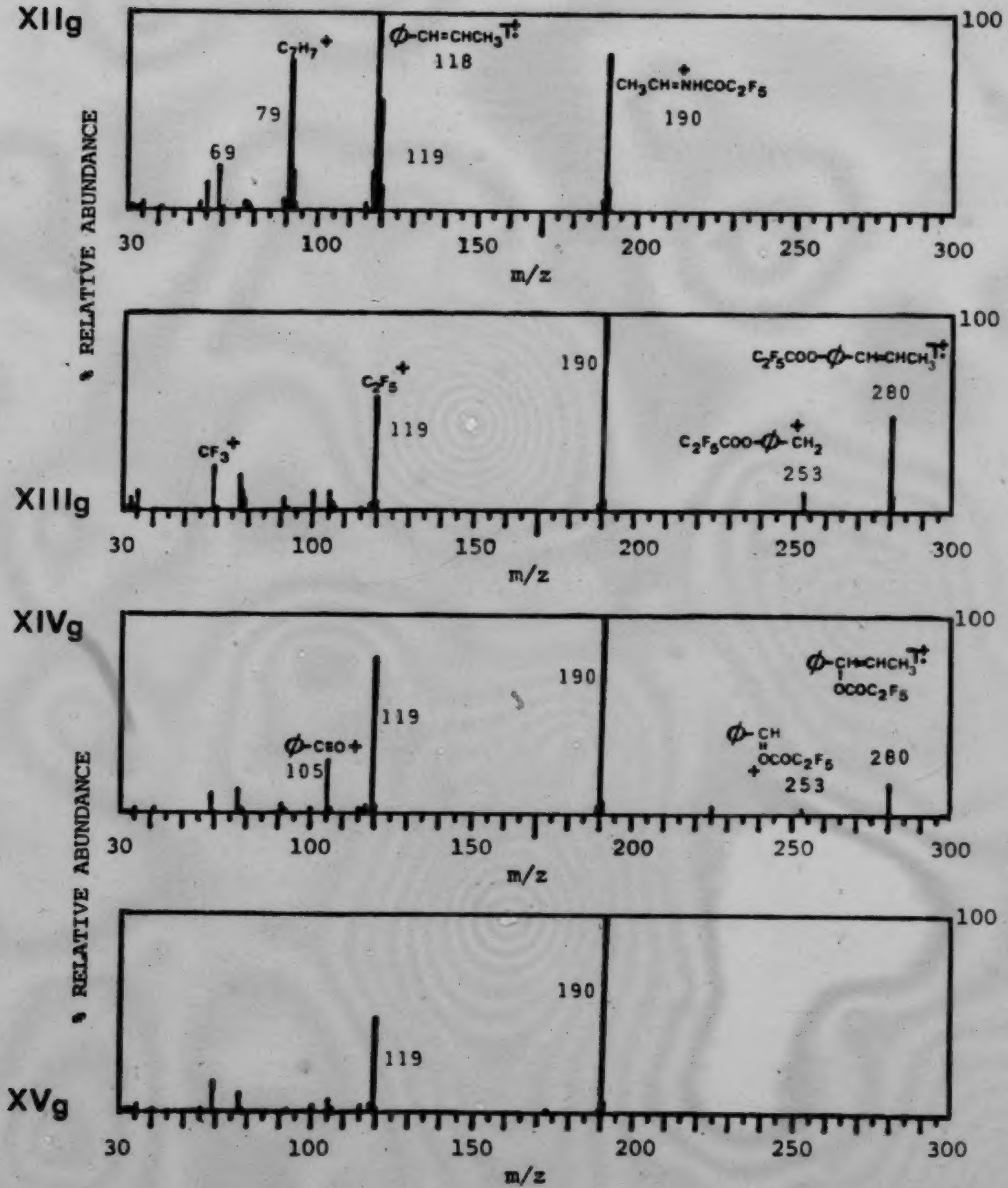
Diagnostic EI-fragmentation ions of the pentafluoropropionylated amines (XIIg, XIVg) are given in Table 23. The PFP-derivatives of p-hydroxyamphetamines (XIIIg) and p— hydroxynorephedrine (XVg) are also provided for comparison. The amine PFP-derivatives gave ion fragmentation patterns only slightly more complex than the parent compounds, and spectral interpretation remained relatively uncomplicated. Mass spectral profiles of all four compounds (XIIg-XVg) were very similar, and characterized by the presence of $CH_3CH = NHCOC_2F_5^+$ (m/z 190), $C_2F_5^{++}$ (m/z 119) and CF_3^+ (m/z 69) ions. In fact, an overall comparison showed a difference of only one or two fragments in their mass spectra (Fig. 47). N-pentafluoropropionyl amphetamine (XIIg) differed from the other spectra by the presence of a phenylpropenyl residue (m/z 118) and a tropylium ion (m/z 91). Derivatives of p-hydroxyamphetamine (XIIIg) and norephedrine (XIVg), on the other hand, had identical mass spectral displays, although there was some variation in

TABLE 23. EI Mass Spectral Data of the Pentafluoropropionyl Derivatives of the Biogenic Amines XII - XV.

COMPOUND	m/z (% RELATIVE ABUNDANCE)[1]
XIIg	281 (absent)$[M^{+\cdot}]$; 190(80)$[CH_3CH{=}NHCOC_2F_5^{+}]$; 119(56)$[C_2F_5^{+}]$; 118(100)$[\mathbf{Ph}\text{-}CH{=}CHCH_3^{+\cdot}]$; 117(20) $[\mathbf{Ph}\text{-}C{=}CHCH_3^{+}]$; 91(76)$[C_7H_7^{+}]$; 69(23)$[CF_3^{+}]$.
XIIIg	443 (absent)$[M^{+\cdot}]$; 280(49)$[C_2F_5COO\text{-}C_6H_4\text{-}CH{=}CHCH_3^{+\cdot}]$; 253(10)$[C_2F_5COO\text{-}C_6H_4\text{-}CH_2^{+}]$; 190(100); 119(58); 105(10)$[\mathbf{Ph}\text{-}C{\equiv}O^{+}]$; 77(18)$[C_6H_5^{+}]$; 69(23). *
XIVg	443 (absent)$[M^{+\cdot}]$; 280(49); 253(3); 190(100); 119(79); 105(27); 77(12); 69(10). *
XVg	605 (absent)$[M^{+\cdot}]$; 190(100); 119(48); 105(7); 77(10); 69(16). *

[1] MASS SPECTRA OF COMPOUNDS XIIg, XIIIg, XIVg, AND XVg GIVEN IN FIGURE 47.

FIGURE 47. Comparison of the EI mass spectra of the pentafluoropropionyl derivatives of amphetamine (XIIg), *p*-hydroxyamphetamine (XIIIg), norephedrine (XIVg), and *p*-hydroxynorephedrine (XVg).



the relative abundances of the fragments. As a result identification of these two compounds had to be supported by GLC retention times.

5.2.2.1.3 Trifluoroacetylation

The use of trifluoroacetic anhydride (TFAA) as the perfluoroacylating reagent was also investigated during the course of the present study. TFA-analogs of the four pentafluoropropionyl derivatives (XIIg, XIIIf, XIVg, XVf) were prepared in the same manner as that used to obtain the PFP-derivatives, and similar products with structures XIIi, XIIIh, XIVi, and XVh were obtained and their structures confirmed by electron-impact mass spectrometry. Diagnostic and abundant ions are shown in Table 24. Fragmentation patterns paralleled those of the PFP-analogs.

Trifluoroacetyl derivatives possessed good chromatographic properties and proved useful for the quantitative analysis of these biogenic amines, but offered no notable advantages over the use of PFP-derivatives. The TFA-compounds, however, appeared marginally less stable. One possible disadvantage to utilizing the trifluoroacetyl derivatives was cited by Anggard and Hankey (522). When amphetamine was used as a test substrate, these authors found that the derivative produced with PFPA gave an EC response some forty times stronger than that obtained with the TFA-amine. Several other studies have similarily demonstrated a

TABLE 24. EI Mass Spectral Data of the Trifluoroacetyl Derivatives of the Biogenic Amines XII - XV.

COMPOUND	m/z (% RELATIVE ABUNDANCE)
XIIIi	231 (absent)$[M^{+\cdot}]$; 140(100)$[CH_3CH{=}NHCOCF_3^{+}]$; 118(85)[**Ph**-$CH{=}CHCH_3^{+\cdot}$]; 117(24)[(m/z 118 - H)$^{+}$]; 91(65)$[C_7H_7^{+}]$; 69(29)$[CF_3^{+}]$.
XIIIh	289 (absent)$[M^{+\cdot}]$; 230(39)$[CF_3COO\text{-}C_6H_4\text{-}CH{=}CHCH_3^{+\cdot}]$; 203(12)$[CF_3COO\text{-}C_6H_4\text{-}CH_2^{+}]$; 175(8)[(m/z 203 - CO)$^{+}$]; 86(100)$[CH_3CH{=}NHCOCH_3^{+}]$; 44(89)$[CH_3CH{=}NH_2^{+}]$; 43(18)$[CH_3C{\equiv}O^{+}]$. *
XIVi	343 (absent)$[M^{+\cdot}]$; 230(19)[**Ph**-$C(OCOCF_3){=}CHCH_3^{+\cdot}$]; 203(18)[**Ph**-$CH{=}OCOCF_3^{+}$]; 182(64) $[CH_3CH{=}N(COCH_3)COCF_3^{+}]$; 175(8)[(m/z 203 - CO)$^{+}$]; 140(87); 105(42)[**Ph**-C≡O]; 77(30)$[C_6H_5^{+}]$; 69(78); 43(100). *
XVh[1]	287 (absent)$[M^{+\cdot}]$; 69(100). *

[1] FRAGMENTATION PROFILE SHOWN IN TABLE 21.

* THE IDENTITIES OF IONS NOT LABELLED ARE GIVEN ELSEWHERE IN THE TABLE.

better ECD response with pentafluoropropionylated compounds to the corresponding TFA-derivatives (525-528). A difference in detection response between derivatizing reagents, however, was not noted in the current study. Minimum EC detection levels, based on a signal to noise ratio of 2:1, were comparable regardless of whether PFPA or TFAA was used.

5.2.2.2 Analysis by GLC

5.2.2.2.1 "Clean-up" Procedure for Biological Samples

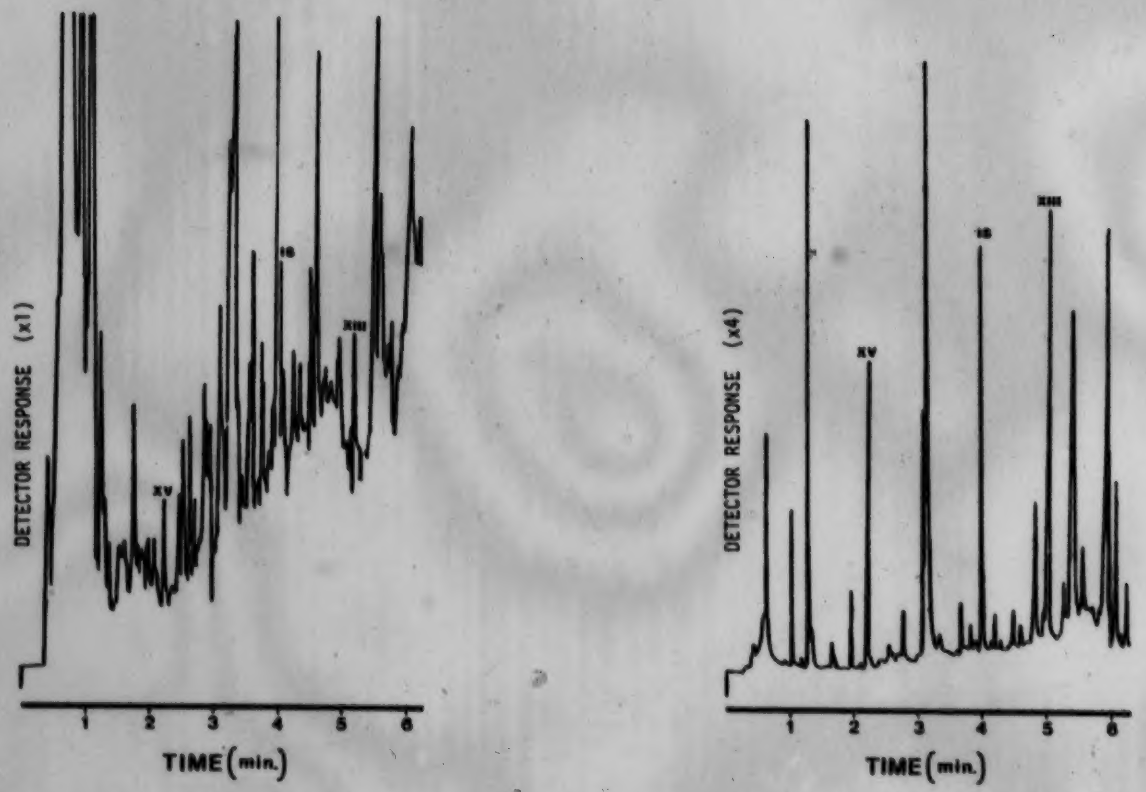
The prevention of most contaminants being carried through the assay procedure and interfering with the analysis by EC-GLC was made possible by means of an initial extraction of the amines into an organic phase; this extraction was accomplished with the ion-pair transfer reagent, di(2-ethylhexyl)phosphoric acid (DEHPA), in chloroform (529). DEHPA, a cationic exchanger, complexes only with compounds containing an amino functional group and removes them from the aqueous phase (530). Once the metabolites (XII-XV) were extracted into the DEHPA solution, attempts were made to perfluoroacylate the amines directly by evaporating the chloroform to dryness and reacting the resulting residue with PFPA. GLC examination of the reaction, however, showed that substantial interference was caused by the presence of the DEHPA and various impurities extracted into the organic solvent. Consequently, it was found beneficial to back extract the amines into 0.5 N HCl, as the lipid soluble contaminants and DEHPA remained in the organic phase. Transfer of the amines into the acid solution was quantitative.

The ion-pair transfer procedure just described was found to be an excellent method for providing "cleaner" aqueous samples for subsequent analysis of the amino compounds (XII-XV), by either Method A or B, as outlined in Section 4.4.2.1.2. Figure 48A shows a typical chromatogram initially obtained by processing a tissue sample prior to inclusion of this liquid ion-exchange step. Detection efficiency for the amines was normally lost below levels of 3 ug/g tissue. In comparison, the chromatogram illustrated in Figure 48B resulted from an identical sample obtained with the ion-exchange step. These EC-GLC traces convincingly demonstrated how utilization of DEHPA significantly improved the chromatographic quality of the assay. By reducing background interference, higher detection senstivities could be used during GLC analysis.

5.2.2.2.2. GLC Column Efficiency

The use of capillary-GLC arose from problems with initial chromatographic efforts utilizing packed columns; these problems included inadequate separation of the derivatives (XIIi, XIIh, XIVi, XVh; internal standard), and tailing of the peaks, particularly at sub-microgram levels.

FIGURE 48. Capillary EC - GLC analysis of the derivatized extract (Method B) of brain tissue obtained from amphetamine treated rats. A) without utilization of 'Clean-up' step involving DEHPA (relative detection response; x1); B) with initial 'Clean-up' step of biological sample (relative detection response; x4).



In comparison, the switch to a capillary column resulted in sharp detection peaks with little or no tailing, and excellent resolution of the compounds (Fig. 48B) The superiority of the capillary column system was further demonstrated by a minimal detection limit of 10 ng/sample.

Calibration curves of the derivatized form of all four amines (XIIi, XIIh, XIVi, XVh) were constructed using capillary EC-GLC and varying quantities of each amine and p— chlorophenylethylamine as the internal standard. All plots were linear over a 10-10 000 ng range.

5.2.2.3 Mass Spectral Analysis

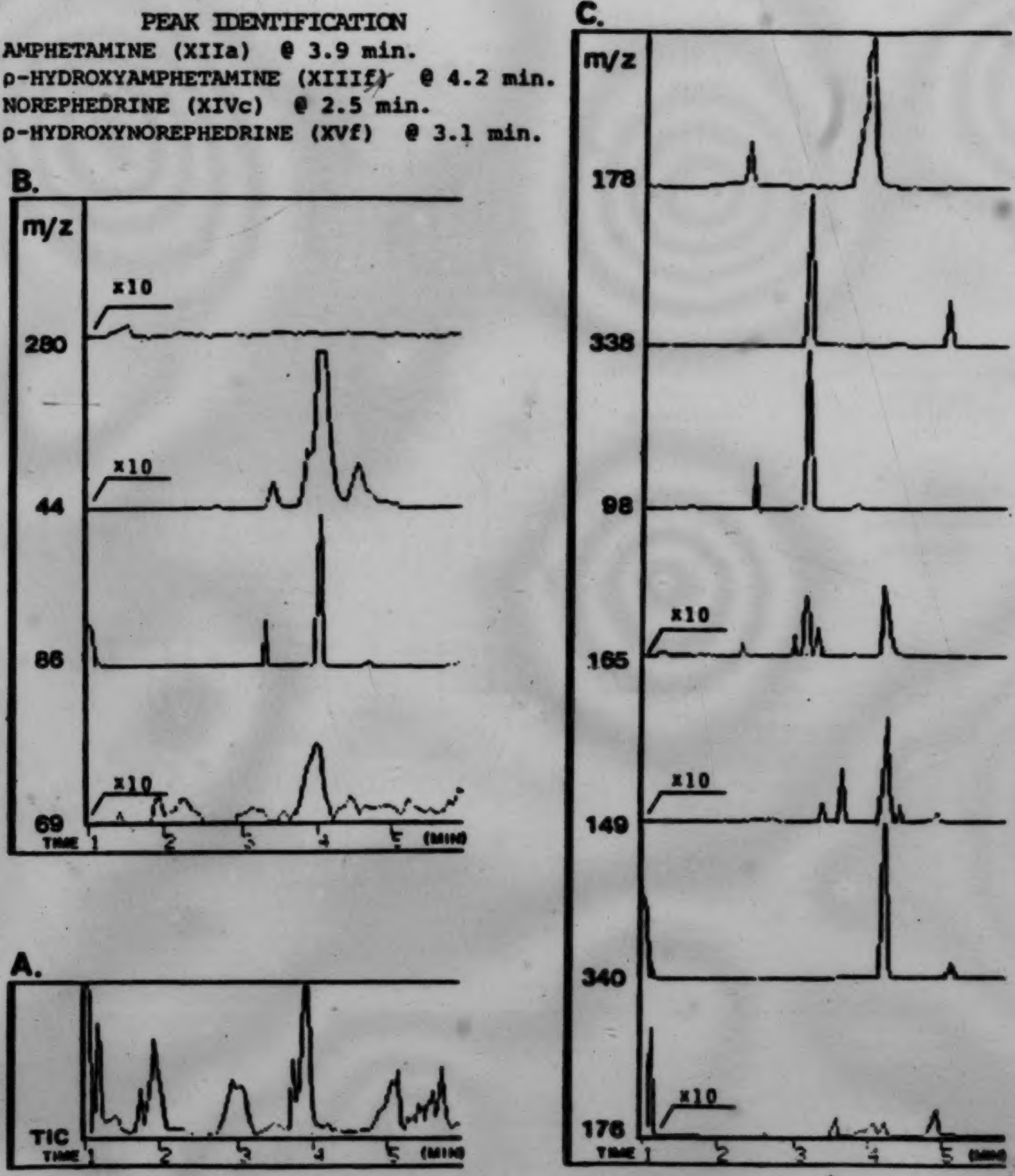
5.2.2.3.1 Total Ion Current (TIC) Spectrometry

A compound eluted from a GLC column cannot be unequivocally identified by comparing its retention time with that of an authentic sample chromatographed under identical conditions. In order to confirm that the chromatographic peaks produced from metabolism samples were in fact representative of the compounds being assayed, mass spectral analyses of the derivatized extracts were carried out. However, due to the extremely low concentrations of the metabolites and the presence of perfluoroacylated endogenous amines which gave fragmentation profiles similar to those of the metabolites, the actual presence of metabolites could not be confirmed based solely on mass spectral data obtained from conventional total ion current (TIC) spectrometry. Furthermore, in some instances, particularly with in vitro samples, the presence of relatively high concentrations of an administered substrate was found to interfere with the detection of trace levels of its metabolites. Whereas the assay procedure (Method B) was developed for EC analysis to avoid this latter problem by allowing amphetamine and norephedrine to degrade into non-interfering, EC - insensitive compounds (XIIa and XIVc, respectively; Fig. 42), TIC detection produced a response for any material entering the ion source. Illustrated in Figure 49A is a typical EI-TIC chromatogram of a processed (Method B) brain extract containing both p- hydroxyamphetamine (XIIIf) and p-hydroxynorephedrine (XVf) as metabolites of amphetamine (XII). As demonstrated, the TIC detector was essentially useless as a selective detector. It is noteworthy that there was no discernible signal for either XIIIf (RT = 4.2 min.) or XVf (RT = 3.1 min.), although amphetamine (XIIa; RT = 3.9 min.) was clearly present. Subsequent attempts to isolate recognizable mass spectra of XIIf or XVf from the TIC trace were unsuccessful.

5.2.2.3.2 Selected Ion Monitoring (SIM)

As an alternative to TIC spectrometry as a means of identification of metabolites, mass fragmentography (selected ion monitoring, SIM) was employed, which provided a means of isolating signals of specific atomic mass units (m/z) from

FIGURE 49. Computer reconstructed chromatograms of a processed brain sample (Method B) obtained from amphetamine treated rats. A) total ion current (TIC) trace using electron-impact MS (EI-MS). B) multiple selected ion monitoring (SIM) using EI-MS. C) multiple selected ion monitoring using chemical-ionization MS (CI-MS).



PEAK IDENTIFICATION

AMPHETAMINE (XIIa) @ 3.9 min.

ρ-HYDROXYAMPHETAMINE (XIIIf) @ 4.2 min.

NOREPHEDRINE (XIVc) @ 2.5 min.

ρ-HYDROXYNOREPHEDRINE (XVf) @ 3.1 min.

the total ion trace. In this manner, the presence of fragments known to be characteristic for certain compounds could be identified, thereby establishing a tentative identification. The greater number of diagnostic ions which could be confirmed, the greater the probability of the presence of the compound being assayed.

5.2.2.3.2.1 Electron-Impact SIM

Initial studies utilizing EI-MS in the single ion monitoring mode revealed a number of insoluble problems. Mass fragmentography requires that the MS instrument be focused on a single intense ion, such as the base peak. However, with perfluoroacylated derivatives the major abundant fragments were usually not characteristic of a single compound. Illustrated in Figure 49B was an attempt to prove, by multiple-SIM, the presence of *p*-hydroxyamphetamine (XIIIf) and *p*-hydroxynorephedrine (XVf) in brain extracts of amphetamine treated rats. The mass spectral data for XVf (Table 22) indicated that its base peak (m/z 69) was the only ion available for scanning as its other diagnostic fragments (m/z 190, 119, 105) had intensities too low to be applicable to SIM. Because suitable ions for monitoring were not available, it was impossible to distinguish a signal characteristic for XVf from background noise (Fig. 49B), especially at the trace level of metabolites encountered during these studies. With *p*-hydroxyamphetamine (XIIIf) the major fragments appropriate for SIM, m/z 86 (100%) and m/z 44 (83%) (Table 22), were also abundant in the mass spectrum of amphetamine (XIIa) (Table 19). Multiple ion monitoring of the GLC eluate at m/z 44 and 86 showed that the expected peak at 4.2 minutes [the retention time of p-hydroxyamphetamine (XIIIf)] was obscured by the amphetamine signal (Fig. 49B). The only other fragment ion in the spectrum of XIIIf possibly suitable for monitoring and not produced by XIIa, m/z 280 (20%), proved too weak to give a discriminating signal. The electron impact induced fragmentation patterns of the acetylated and/or perfluoroacylated derivatives of amines XII-XV yielded inadequate mass spectral characteristics for SIM. As a result, very little information was gained utilizing this detection mode for the analysis of metabolic samples.

5.2.2.3.2.2. Chemical-Ionization SIM

In comparison to the inadequate selectivity and ensuing poor sensitivity obtained with EI-SIM, it was found that these problems could be overcome through the utilization of chemical ionization - SIM (CI-SIM) (Fig. 49C). As illustrated, CI-induced fragments diagnostic for p-hydroxyamphetamine (XIIIf; m/z 340, 149, 165) and for p-hydroxynorephedrine (XVf; m/z 338, 165, 98) were resolved clearly, at the correct GLC retention times, strongly indicating the presence of both phenolic compounds (XIII, XV) as metabolites of amphetamine (XII). Levels of detection with CI-SIM were at least comparable to those obtained with EC-detection. Attempts to show that norephedrine (XIV) was a metabolite of XII were made by monitoring for the CI-induced base fragment (m/z 176) of XIVc, but were unsuccessful (Fig. 49C).

A further indication of why the SIM procedure was favored in CI-MS was the absence of interference arising from either background or high levels of substrate. As demonstrated in Figure 49C, scanning the base peak of the amphetamine derivative XIIa (m/z 178 [MH^+]), gave a response which was sufficient to routinely create a voltage overload of the detection system. This prompted activation of a safeguard mechanism, which, by forcing a drop in the electron multiplier voltage (sensitivity), effectively blocked out further monitoring of the ion current at m/z 178. Yet even at this relatively high concentration of amphetamine, detection of its metabolites was not obstructed as would have been the case with EI-SIM. Multiple selective ion detection of the p-hydroxyamphetamine derivative (XIIIf) was still possible despite being concealed under the amphetamine signal. The output for the selected ions at masses m/z 340, 165 and 149 still demonstrated optimum response.

An important advantage in utilizing CI-MS for SIM was that a clearly discernible spectrum for each compound was obtained. In contrast, use of EI-MS produced spectra which displayed similar, sometimes indistinquishable, fragmentation profiles for more than one amine derivative. As a result, chemical ionization was able to provide for at least one very diagnostic fragment of intense relative abundance for SIM. CI-induced fragmentation profiles of the possible amine derivatives resulting from assay procedures A (XIIa, XIIIf, XIVc, XVf) and B (XIIg-XVg) are given in Table 25. Most compounds, except for the directly pentafluoropropionylated derivatives of norephedrine (XIVg) and p-hydroxynorephedrine (XVg), gave quasimolecular ions (MH^+) as their base peak. The major fragment of the two PFP-alcoholamines (XIVg, XVg) was the MH^+-164 $[(M\text{-}HOCOC_2F_5)^+]$ ion (Fig. 50). This formation of a styrene-type ion was analogous to the apparent loss of H_2O displayed by the N,O-diacetate derivative of p-hydroxynorephedrine (XVa; Fig. 41).

5.2.3 Rat Brain Metabolism of Amphetamine and Norephedrine In Vivo and In Vitro

The levels of substrates and metabolites found in whole brain tissue after ip. injection of rats with equimolar quantities of each amine are given in Table 26. The concentration of amphetamine (XII), p-hydroxyamphetamine (XIII), and p-hydroxynorephedrine (XV) present following administration of amphetamine are comparable to values obtained in other studies (151, 156, 160, 178, 181). Rat brain levels of XIV and XV after ip. injection of norephedrine (III), of p-hydroxynorephedrine (XV) after its

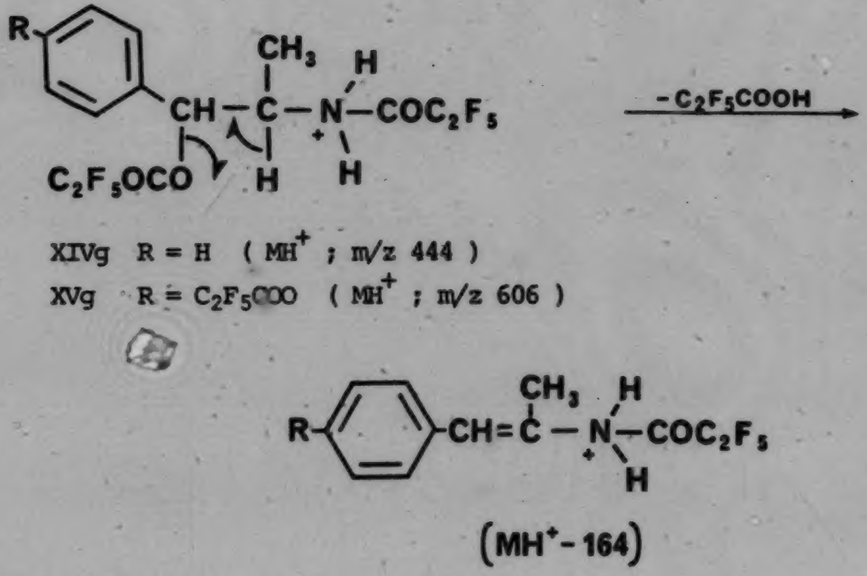
Table 25. Chemical Ionization Mass Spectral Data of the Amine Derivatives Obtained Utilizing the Procedures Developed for the Analysis of Trace Amines in Biological Fluids.

COMPOUND	MOLECULAR WEIGHT	m/z (% RELATIVE ABUNDANCE)
PROCEDURE A[1]		
XIIa	177	218(7)[M+41$^+$]; 206(30)[M+29$^+$]; 178(100)[M+1$^+$].
XIIIf	339	380(6)[M+41$^+$]; 368(18)[M+29$^+$]; 340(100)[M+1$^+$]; 194(14); 165(27); 149(20).
XIVc	175	216(13)[M+41$^+$]; 204(30)[M+29$^+$]; 176(100)[M+1$^+$]; 135(40); 134(84).
XVf	337	378(12)[M+41$^+$]; 366(24)[M+29$^+$]; 338(100)[M+1$^+$]; 297(10); 165(12); 98(44).
PROCEDURE B[2]		
XIIg	281	322(6)[M+41$^+$]; 310(12)[M+29$^+$]; 282(100)[M+1$^+$]; 165(34); 129(18); 119(24); 117(15).
XIIIg	443	484(6)[M+41$^+$]; 472(12)[M+29$^+$]; 444(100)[M+1$^+$]; 280(8).
XIVg	443	472(4)[M+29$^+$]; 444(2)[M+1$^+$]; 280(100); 190(4).
XVg	605	634(3)[M+29$^+$]; 606(2)[M+1$^+$]; 442(100); 190(13); 165(10).

[1] ACYLATION → EXTRACTION → HYDROLYSIS → PENTAFLUOROPROPIONYLATION

[2] EXTRACTION → PENTAFLUOROPROPIONYLATION

FIGURE 50. Formation of the chemical-ionization induced base fragment of the pentafluoropropionyl derivatives of norephedrine (XIVg) and *p*-hydroxynorephedrine (XVg), as produced by the loss of pentafluoropropionic acid from the quasimolecular ion (MH+).


R
CH₃
H
CH
C
N
COC₂F₅
H
H
C₂F₅OCO
−C₂F₅COOH
R
CH=C
CH₃
H
N
COC₂F₅
H
(MH⁺-164)

XIVg R = H (MH^+ ; m/z 444)

XVg R = C_2F_5COO (MH^+ ; m/z 606)

XIVg (m/z 280)

XVg (m/z 442)

TABLE 26. Rat Whole Brain Levels of Amphetamine (XII), Norephedrine (XIV), and Their *para*-Hydroxylated Metabolites [*p*-Hydroxyamphetamine (XIII); *p*-Hydroxynorephedrine (XV)].

SUBSTRATE [2]	METABOLITE as ηmol/g BRAIN TISSUE ± S.D.[1]			
	AMPHETAMINE	*p*-HYDROXYAMPHETAMINE	NOREPHEDRINE	*p*-HYDROXYNOREPHEDRINE
AMPHETAMINE	67.05 ± 4.50 (3)[1]	0.7556 ± .0934 (15)	ND.[3] (3)	0.4976 ± .0760 (12)
p-HYDROXYAMPHETAMINE	—	0.4960 ± .0735 (13)	—	0.3180 ± .0449 (13)
NOREPHEDRINE	—	—	41.17 ± 4.05 (3)	0.7886 ± .0970 (11)
p-HYDROXYNOREPHEDRINE	—	—	—	0.4844 ± .0557 (9)

[1] NUMBER OF EXPERIMENTS GIVEN IN PARENTHESIS ().

[2] SUBSTRATE CONCENTRATION, 74.0 umol/kg (FREE BASE) INTRAPERITONEALLY (*ip.*).

[3] ND. - NOT DETECTED.

ip. injection, and of XIII and XV after *ip*. administration of *p*-hydroxyamphetamine (XIII) have apparently not been determined previously.

Brain levels of *p*-hydroxyamphetamine (XIII) (.7556 nmol/g), resulting from the administration of amphetamine (XII) were significantly higher ($p < 0.025$; student's t-test) than those obtained (.4960 nmol/g) when XIII itself was injected. Thus, even if all the administered amphetamine (XII) had been *para*-hydroxylated in the liver (and this had not occurred - see Table 26), it would not account for the levels of *p*-hydroxyamphetamine (XIII) detected. These results indicated that although *p*-hydroxyamphetamine (XIII) was capable of permeating into the brain to a limited extent, at least a portion of the XIII detected was due to the *para*-hydroxylation of amphetamine occurring in rat cerebral tissue. Similar results were observed when norephedrine (XIV) was the substrate. Although .4844 nmol/g of *p*-hydroxynorephedrine (XV) was measured in brain tissue after the *ip*. injection of XV, this did not fully account for its significantly higher ($p < 0.01$) brain levels (.7886 nmol/g) after injection of norephedrine (XIV).

The view that *para*-hydroxylation can occur in brain tissue agreed with certain other observations. Costa and Groppetti (157) reported that pretreatment of rats with α – methyltyrosine significantly decreased the amount of *p*-hydroxyamphetamine (XIII) found in the brain following administration of amphetamine (XII), even though neither the amount of amphetamine reaching the brain, nor the amount of p-hydroxyamphetamine produced by the liver changed. Kuhn et al. (161) could not detect p-hydroxyamphetamine (XIII) in brain after ip. injection of amphetamine (XII), but found substantial levels following administration of XII intracisternally (ic.). But whereas Kuhn et al. felt this was sufficient evidence to show para-hydroxylation did occur in the brain, Freeman and Sulser (152) concluded from similar results obtained with ic. injections that amphetamine had been transported from the CSF to the liver and metabolized to XIII which in turn was taken up by the brain.

Attempts to show that norephedrine (XIV) was an in vivo rat brain metabolite of amphetamine (XII) were unsuccessful (Table 26). If any XIV was so formed, it was in an amount below the detection limit of the assay. This failure to detect XIV in rat brain tissue agreed with previous reports (148, 162, 170). However, in contrast, Lewander (178) was able to show the presence of extremely low levels of XIV-^{14}C (< .066 nmol/g tissue) in rat brain following the ip administration of 20mg/kg carbon-labeled amphetamine (XII-^{14}C). Kuhn et al. (161) also found appreciable amounts of norephedrine (XIV) following the ic. injection of amphetamine (XII). But while rat brain tissue was shown in the current study to have no ability to beta— hydroxylate amphetamine, p-hydroxyamphetamine (XIII) was actively converted to p-hydroxynorephedrine (XV) (Table 26). Assuming XV was formed completely from XIII (no contribution *via* p-hydroxylation of norephedrine), approximately 39 % of XIII which accumulated in brain tissue following the administration of either amphetamine (XII) or p-hydroxyamphetamine (XIII) underwent *beta*-hydroxylation. Due to the extremely low β-hydroxylating activity found in rat liver (513, 531) and because of the amphoteric nature of p-hydroxynorephedrine (XV) making it difficult to cross the blood brain barrier, it was unlikely that any XV produced by hepatic enzymes contributed significantly to the level of XV measured in the brain.

It is very possible that dopamine β-oxidase, the enzyme responsible for the conversion of dopamine to noradrenaline and m-tyramine to m-octopamine, is also responsible for the *in vivo* β-hydroxylation of p-hydroxyamphetamine (XIII). This enzyme has been shown to catalyze the *in vitro* β-hydroxylation of various phenylethylamines (170, 176, 179, 525, 532). An interesting finding of these studies was that *in vivo*, amphetamine was not an active substrate whereas p-hydroxyamphetamine was (170, 532). By having a hydroxyl group in the *para*-position, p-hydroxyamphetamine resembled other natural substrates (dopamine, tyramine) for β-hydroxylase, and presumably permitted better access to the enzyme (533).

In vitro studies using rat brain 10 000Xg homogenate supernatant confirmed that both para- and beta-hydroxylating enzymes are present in brain tissue (Table 27). Metabolism of amphetamine (XII) (1.0 umol) produced trace amounts of p-hydroxyamphetamine (XIII) (.74 nmol) and p-hydroxynorephedrine (XV)(.21 nmol). The incubation of XIV (1.0 umol) similarly produced XV (.53 nmol) by p-hydroxylation. The β-hydroxylation of XIII (1.0 umol) to XV (1.11 nmol) was also demonstrated.

In most studies involving extrahepatic xenobiotic metabolism, the activity demonstrated in brain has been relatively low. It appears that the metabolism of amphetamines (XII) in brain tissue is associated with specific enzyme systems which normally utilize endogenous amines. The low extent to which biotransformation occurs probably also reflects the poor affinity of XII for these systems. This was well illustrated in the current study in which the enzyme(s) involved in in vivo beta-hydroxylation preferred p-hydroxyamphetamine as the substrate since p-hydroxynorephedrine was produced in a much greater yield than norephedrine, in spite of the much higher levels of amphetamine available for conversion in the brain (Table 26).

The presence of ring hydroxylating enzyme systems involved in catechol production from natural substrates has recently been established in brain tissue (534, 535), but whether these enzyme systems are involved in the p-hydroxylation of amphetamines remains to be determined.

TABLE 27. *In Vitro* Rat Brain Metabolism of Amphetamine (XII), *p*-Hydroxyamphetamine (XIII), and Norephedrine (XIV).

SUBSTRATE [2]	METABOLITE as ηmol/g BRAIN INCUBATION ± S.D.[1]			
	AMPHETAMINE	*p*-HYDROXYAMPHETAMINE	NOREPHEDRINE	*p*-HYDROXYNOREPHEDRINE
AMPHETAMINE	∞[3]	0.7444 ± .1205 (10)[1]	ND.[4] (5)	0.2108 ± .0491 (10)
p-HYDROXYAMPHETAMINE	---	∞[3]	---	1.1174 ± .1210 (6)
NOREPHEDRINE	---	---	∞[3]	0.5330 ± .0910 (6)

[1] NUMBER OF EXPERIMENTS GIVEN IN PARENTHESIS ().

[2] SUBSTRATE CONCENTRATION, 1.0 μmol (ADDED AS FREE BASE).

[3] RECOVERY OF SUBSTRATE NOT DETERMINED.

[4] ND. - NOT DETECTED.

5.3 ISOLATED HEPATOCYTES IN DRUG METABOLISM STUDIES

5.3.1 Studies on Isolated Hepatocytes

5.3.1.1 Isolation of Adult Rat Liver Parenchymal Cells

5.3.1.1.1 Preparation of Liver Cell Suspensions

Of the several methods available, the most efficient yield of intact, viable hepatocytes was obtained by the sequential treatment of rat liver reported in Section 4.3.3.2.1. Hepatic cells were derived from mature rats by a modification of the two-step liver perfusion technique reported by Seglen (256), who employed a washout of endogenous Ca^{++} with a Ca^{++}-free buffer, followed by Ca^{++} re-addition combined with enzyme treatment. In the modified procedure, Ca^{++} removal prior to enzyme treatment was assisted by a second perfusion of the liver *in situ* with an EDTA/EGTA- containing buffer. This resulted in a marked improvement in the ease and extent of liver dispersion compared to when Seglen's method was tested, and appeared to be a direct consequence of the efficiency of Ca^{++} removal. Several studies have confirmed that removal of endogenous Ca^{++} is best achieved by the use of chelating agents rather than by just a simple washout with buffer (260, 326). The initial perfusion procedure with the Ca^{++}-free buffer (Buffer A) was retained, however, in order to facilitate complete washout of blood from the organ. Jeejeebhoy *et al.* (290, 319) have reported that the proper elimination of all blood is vital for a good cell yield and needs to be performed carefully prior to any enzyme perfusion.

Attempts to disrupt the liver without prior removal of Ca^{++} resulted in the tissue remaining essentially intact, and few viable cells were recovered. Those cells which were obtained were usually in clumps and inevitably damaged. This demonstrated that initial removal of endogenous Ca^{++} was an unquestionable requirement for thorough dispersion of liver tissue by enzymic treatment. The effectiveness of collagenase without the re-addition of Ca^{++} was not tested.

It has been demonstrated by others that the presence of certain chelating agents inhibited the activity of Ca^{++}-dependent enzymes such as collagenase (260, 326, 536). In the current procedure this necessitated the removal of any remaining EDTA and EGTA found in the liver after perfusion with the chelating buffer (Buffer B). Their elimination was accomplished by a rapid washout of the liver with Buffer A just prior to enzyme treatment. Failure to include this additional perfusion step significantly decreased the yield of intact cells obtained, presumably due to inhibition of the collagenase.

5.3.1.2 Purification of Liver Parenchymal Cells

Examination by light microscope of the initial, crude cell suspension (Plate 1), revealed not only individual intact hepatocytes, but also scattered cell clumps, various non-parenchymal liver cells, fragments of connective tissue, damaged cells and subcellular debris. Purification of the preparation to remove these contaminants was achieved by a combination of filtration and differential centrifugation. Microscopic examination of the final suspension (Plate 2) showed it to contain spherical cells of an approximately uniform size, indicating the majority of hepatocytes were diploid in nature. Based on cell size and phase-contrast microscopy, however, a noticeable portion of the parenchymal cells was also in the tetraploid state, as well as the occasional octaploid form (537-589). Some smaller cells were also observed and were presumed to be of endothelial origin. These non-parenchymal cells constituted only a small portion of the total cell yield and were not included in the cell count.

5.3.1.3 Cell Yield

At optimum conditions, the number of hepatocytes recovered from a 200-250 g rat was 280-340 x 10^6 cells, as determined by use of a hemocytometer. Based on the ability of the cells to exclude trypan blue dye, the viability index

PLATE 1. Photomicrograph of the isolated adult rat liver cells, as the initial crude cell suspension (35x magnification). Cells were prepared by enzymic dispersion of the liver according to the perfusion procedure described in Materials and Methods. (Bar = 100 μm)

PLATE 2. Final purified suspension of isolated adult rat liver parenchymal cells (hepatocytes) (35x magnification), obtained by the low speed centrifugation of the initial crude liver cell suspension. (Bar = 100 μm)

was consistently 85-90%. Thus, the viable cell yield was calculated at approximately 270 x 10^6 cells per experiment.

Although it was impossible to weigh the isolated cells, calculations based on liver weights of rats of similar body weights gave a yield of about 65 x 10^6 cells/g liver (wet tissue). Laishes and Williams (288) reported that the number of parenchymal cells found in rat liver was approximately 140 x 10^6 cells/g tissue. Based on this estimation, the number of isolated, intact hepatocytes recovered, accounted for about 46% of the total possible cell number.

5.3.2 Primary Monolayer Cultures

5.3.2.1 Culture Morphology

The behavior of isolated hepatocytes seeded on collagen-coated petri dishes appeared reproducible once a routine procedure was developed. The cells readily attached to the substratum within 4-6 hours after plating and began to flatten out (Plate 3). By 24 hours, cells had formed contacts with each other, taking on polygonal shapes and developed into islands of epithelial-type sheets (Plate 4). The following 24-96 hours showed progressive spreading of the cytoplasm into a thin monolayer, during which time the cells starte[illegible] to become less polygonal and to lose their definitive shape (Plate 5). After approximately 8-10 days, the primary culture began to show an easily observable morphological deterioration. Cells, demonstrating a slow, progressive

PLATE 3. Adult rat hepatocytes 3-5 hours after isolation and their immediate seeding upon collagen coated plates (35x magnification). Attached and flattened cells display the initial stages of monolayer formation. Cells were cultured as described in materials and Methods. (Bar = 100 µm)

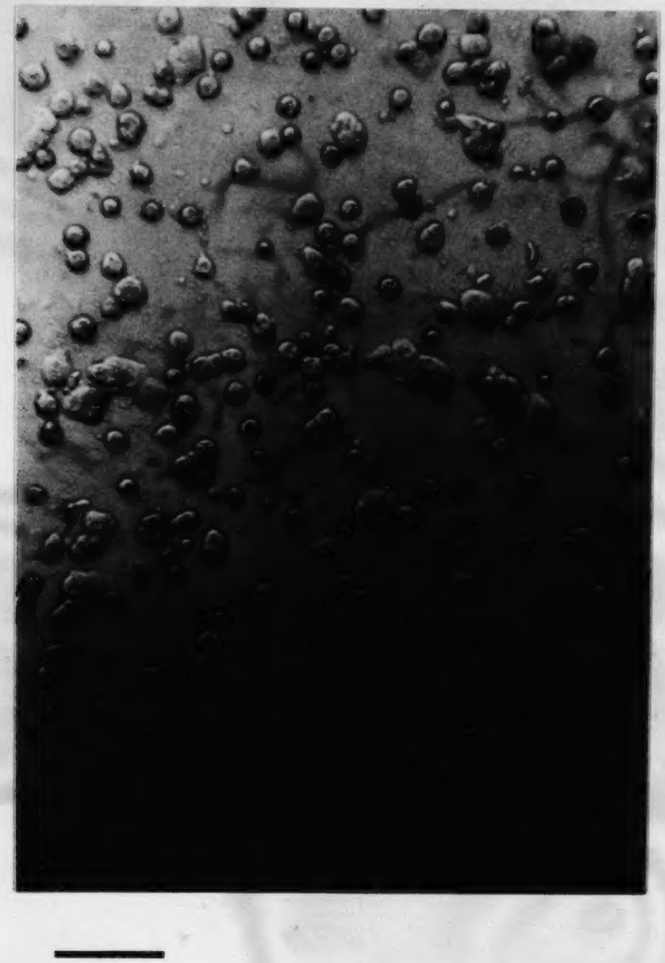


PLATE 4. Primary culture of adult rat hepatocytes 24 hours post-inoculation. The epithelial-like cells which have securely anchored to the collagen substratum have formed tight lateral contacts with each other and have aquired a polygonal morphology. Note presence of large population of binucleated cells. A) 35x magnification (Bar = 100 µm); B) 350x magnification (Bar = 16 µm).

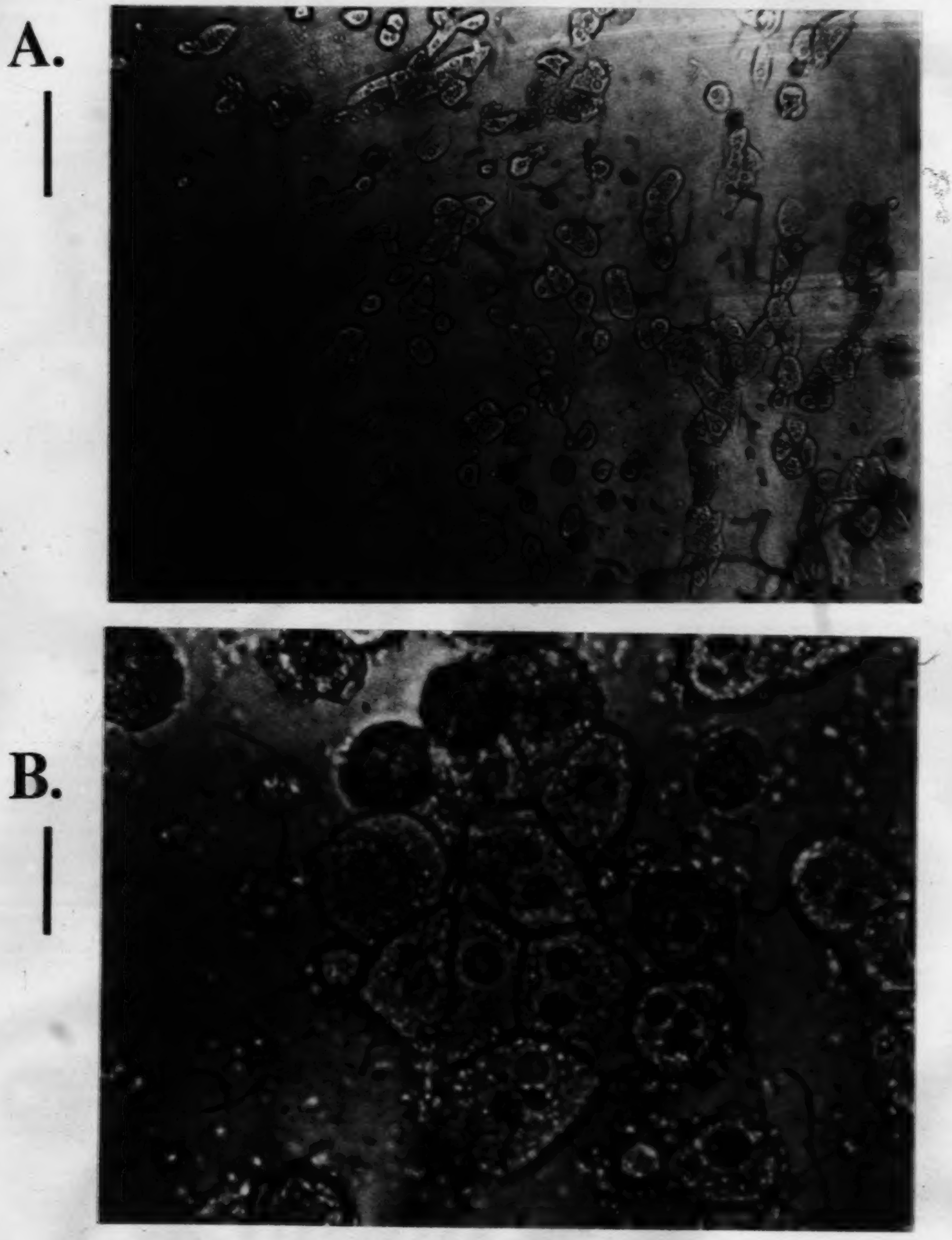
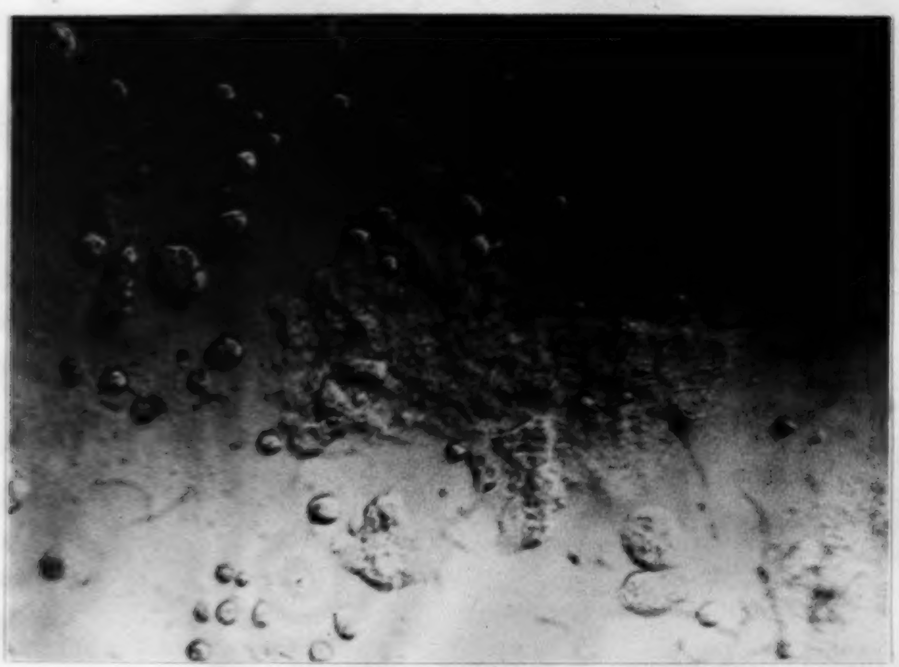
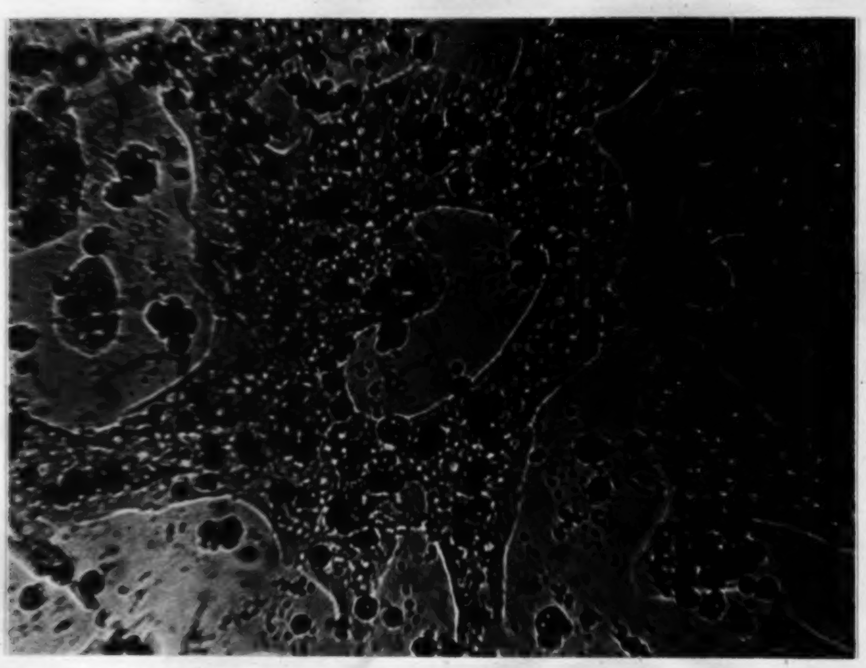


PLATE 5. Primary culture of adult rat hepatocytes plated on a thin collagen layer (TCL) substratum. A) 48 hours post-inoculation (35x magnification; bar = 100 µm). Note spreading of cells to establish a monolayer morphology. B) 120 hours post-inoculation (35x magnification, photographed under increased light; bar = 100 µm). Cells cultured on this type of collagen substratum have lost most of their definitive shape by this time. Cells that are out of focus may appear dark in this picture, but are structurally intact (trypan blue exclusion test).

A.



B.



structural degeneration, either disintegrated leaving "ghosts" distributed throughout the substratum (Plate 6), or became rounded and eventually detached from the support. Increasing cell death became apparent by the presence of floating cellular debris. Hepatocytes under these conditions could be maintained for approximately two weeks, at which time only sporadic islands of cells in monolayer formation could be observed.

In all the studies carried out, no evidence was obtained to support the possibility that proliferation of mature rat hepatocytes had occurred to any extent. In several instances where rapid growth of cells was observed in 3-5 day cultures, the presence of fibroblasts was easily identified by their stellate shape (Plate 7). This overgrowth of the parenchymal cells was a direct result of the improper removal of the endothelial cells from the hepatocyte suspension.

5.3.2.2 Cell Attachment and Survival

Studies were initially performed to compare the attachment efficiency of primary hepatocytes plated on glass, tissue culture, plastic or collagen-coated petri dishes. Because of the favorable results obtained when a thin film of collagen was used as the attachment support, subsequent evaluations were carried out on the survival of cells cultured on three variations of the collagen substratum;

PLATE 6. Primary monolayer culture of adult rat hepatocytes 10 days in culture (350x magnification; bar = 16 μm). The majority of cells have displayed a loss of structural integrity by this time. Note presence of 'ghosts' of cells which had disintegrated earlier in culture.

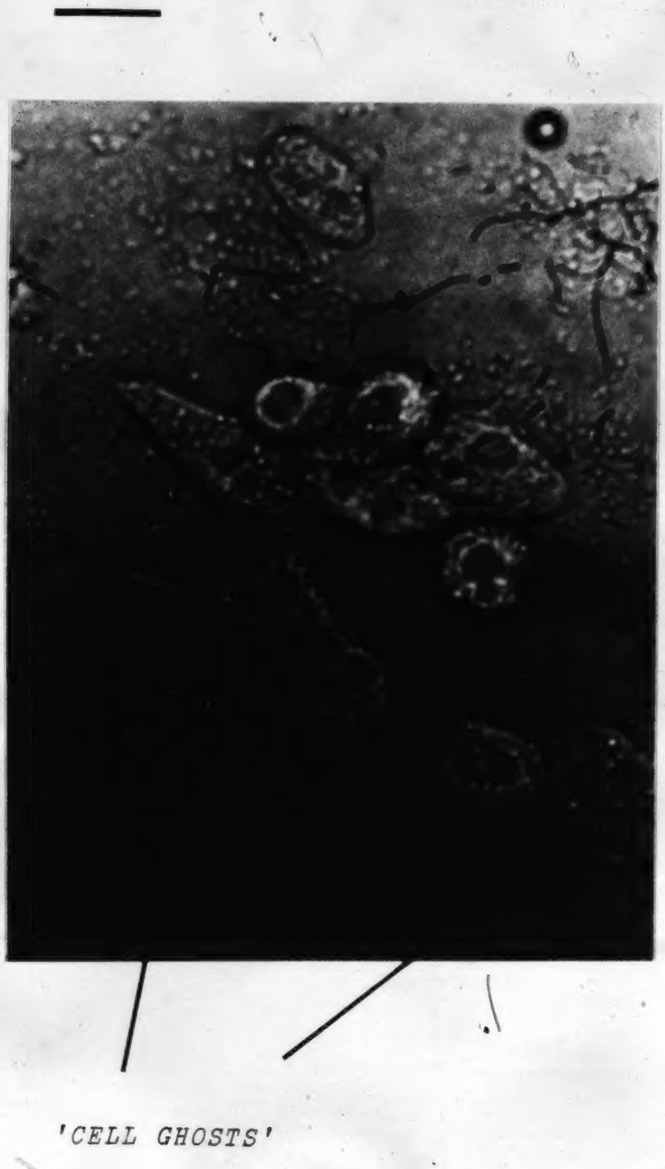


PLATE 7. Primary culture of adult rat hepatocytes 6-8 days post-inoculation, showing a significant number of fibroblastoid-like cells (stellate-shaped) spreading on the substratum (collagen gel) suface. (100x magnification; bar = 35 µm).

'FIBROBLASTOID CELLS'

thin collagen layer (TCL), collagen gel (CG), and floating collagen membrane (FCM).

5.3.2.2.1 Glass, Plastic and Collagen Substrata

Results from investigations performed demonstrated that the use of rat tail collagen as an attachment support markedly enhanced the overall survival of adult rat liver parenchymal cells in vitro (Table 28, Fig. 51). Initial findings showed that glass had a very low attachment capability (Table 28). In comparison, cellular attachment was significantly higher when plastic plates were used, although the extent to which cells further established contact between themselves was very limited. Arrangement into monolayer morphology did not occur on plastic. Apparently cell contact and monolayer formation can only occur when proper adhesion of the cells takes place on a physiologically relevant substratum (346) and the consequence of being unable to establish proper contact with the support was displayed by hepatocytes cultured on plastic plates. Regardless of the fact that these cells were able to initially anchor themselves to the plastic, they still underwent rapid deterioration. Survival of cultures on plastic was limited to 1-2 days.

As a further examination of cell instability, hepatocytes which did not initially adhere to the plastic (or glass) substratum were removed by gentle washing at times 2,

TABLE 28. Attachment Efficiency of Adult Rat Hepatocytes Plated on Various Substrata.

SUBSTRATUM	RELATIVE[1,2] ATTACHMENT EFFICIENCY (%)	CULTURE[3] SURVIVAL TIME (days)
GLASS	<1	<1
PLASTIC	25 - 30	1 - 2
THIN-COLLAGEN FILM	50 - 60	~14
COLLAGEN GEL	50 - 60	~16
FLOATING COLLAGEN MEMBRANE		~23

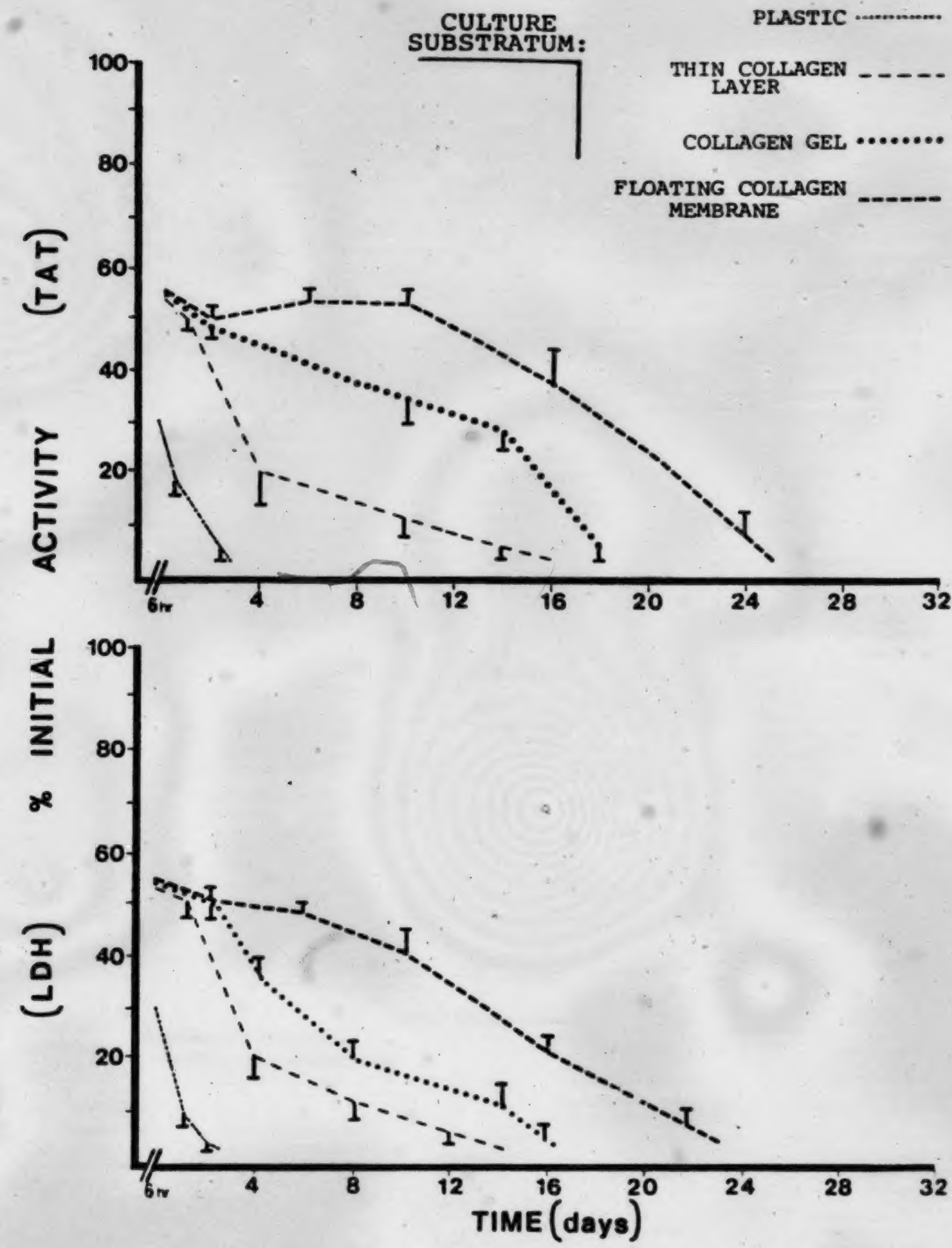
[1] AFTER 6 HOURS INCUBATION TIME.

[2] SEEDING DENSITY 2×10^6 cells/plate.

[3] MEDIUM CHANGED EVERY 48 HOURS.

Cultures were maintained for 6 hr. and the plates rinsed with incubation medium. The cells which attached to the glass and plastic plates were removed with a 0.1% collagenase/0.25% trypsin solution in PBS. The recovered cells were counted, and viability determined by trypan blue exclusion. In order to determine the attachment efficiency on collagen plates, viable cells were tested for LDH levels, and compared to: i) LDH levels obtained from cells on plastic plates where cell quantities were known, and ii) per cent level of LDH activity of freshly isolated cells.

FIGURE 51. Culture survival times of adult rat hepatocytes plated on various substrata, in the presence of DMEM/F-12 media, insulin and FBS. [Based on lactate dehydrogenase (LDH) and tyrosine aminotransferase (TAT) activities measured in viable cells] The points represent the means and standard deviations of at least 5 measurements per time period obtained from at least 2 separate experiments.



4 and 8 hours post-inoculation and tested for viability by dye exclusion. The number of cells capable of incorporating tryphan blue rose from 20% at two hours, to approximately 30% at four hours, to a high of 75% at eight hours. This supported the earlier contention that cells unable to develop secure contact with a solid surface remained structurally unstable.

In contrast to the use of glass and tissue plastic, culturing hepatocytes on a thin film of collagen, as adopted from the studies of Michalopoulos and Pitot (346), proved far superior in attachment efficiency, monolayer formation and survival time (Table 28). Under optimum conditions, 45-55% of the cells were attached after 6 hours and measurable enzyme activites (LDH, TAT) were observed for up to two weeks. Apparently, the mechanism by which adhesion to plastic occurs is different from that of attachment to collagen and does not promote continuing monolayer formation (540, 541). Mere attachment of rounded cells to the support is not sufficient for continued survival. Still, the exact relationship between the ability of rat hepatocytes to establish themselves on collagen and the anchorage of liver parenchymal cells in vitro is unclear. There is strong evidence though, to suggest that laminin (a basement membrane protein) found in collagen, mediates the attachment and spreading of cells in vitro, and that hepatocytes may be attached to this protein in vivo (298, 308, 351).

5.3.2.2.2 Connective Tissue Matrix: Collagen and the Significance of Monolayer Formation

The improved findings noted when a thin layer of collagen was employed as an anchorage substratum prompted a further look at two other forms of collagen support, a collagen gel (CG) and a floating collagen membrane. Hepatocytes were plated on all three collagen supports and variations were compared with respect to survival times of the cells and the appearance of the cultures as examined under light microscope.

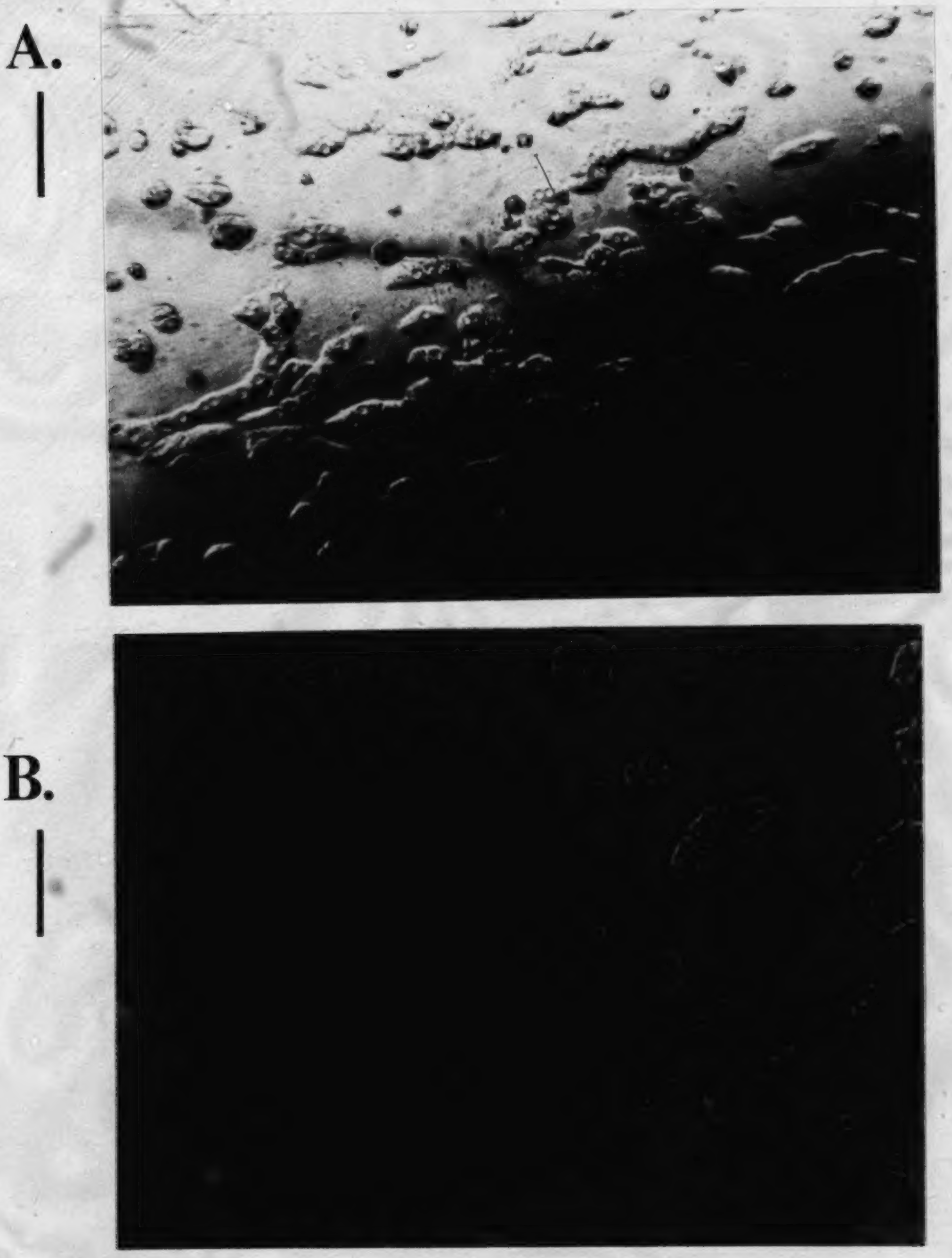
During the first several hours following the plating of hepatocytes on collagen gel, the cells underwent the same characteristic behavior as cells inoculated on the thin collagen coating. There was no difference in the cellular attachment efficiency between TCL and CG (Table 28), and both collagen forms were able to support subsequent development of the hepatocytes into primary monolayer cultures. But while the overall duration of culture viability was also similar between TCL and CG supports (14 days vs. 16 days, respectively), enzyme activity at specific time intervals was greater with the gel support (Fig. 51). In direct comparison though, the longest survival times were obtained with hepatocytes cultured on a floating collagen membrane. Floating membrane supports (FCM) were obtained from cultures set up on collagen gels by detaching the gel from the surface of the petri dish at 12 hours post-inoculation. By allowing the membrane to float freely in the medium, it undergoes a gradual shrinkage in size from its original diameter of 60 mm to approximately 10 mm after several days incubation time. This shrinkage was attributed to the continuing organization of the hepatocytes into a monolayer conformation, since the membrane retains its original size if incubated in the absence of viable cells. As a result of the greater flexibility of this support, the appearance of hepatocytes grown on the FCM no longer resembled cells cultured on the more rigid substrata (TCL,CG). Cells were smaller and appeared thicker and more cubical than the flattened, stretched appearance characteristic of hepatocytes in a normal, spreading monolayer culture. Between approximately three to seven days in culture, contraction of the membrane allowed increased contact between hepatocytes, resulting in formation of small plate-like structures 2-5 cells thick. Extensive aggregation of cells did not occur. Due to the nature of the floating membrane, a more detailed microscopic or photographic examination of these cultures was not possible. No further changes in culture morphology were noted until deterioration of either the cells or support became evident.

The variations in enzyme activity measured in hepatocytes grown on the modified collagen supports appeared to be a reflection of the morphological differences between the cell populations. This change in morphology was probably due to the adaptation of the cells to the attachment support, and furthermore, the geometric configuration of a cell may take precedence over other factors such as hormonal activity or serum growth factors in controlling survival.

Primary monolayers formed on the thin collagen layer (TCL) and collagen gel (CG) are shown in Plates 5 and 8, respectively. Cultures on TCL displayed extensive spreading, with hepatocytes eventually losing any definitive shape. In comparison, monolayers on the collagen gel tended to be confined to smaller islands of cells which maintained their individual polygonal shapes for a longer duration. Sattler et al. (353) proposed that retention of the polygonal form was essential for prolonged cell survival in vitro, as it signified that the ultrastructure of cytoplasmic organelles remained intact. This was consistent with the current interpretation that extensive spreading of the cells during monolayer formation, resulting in loss of the cells polygonal structure, may actually impede survival.

Further evidence to suggest that extensive monolayer formation was not an absolute requirement for maintaining hepatocytes in vitro was obtained from several sources. One such indication was that hepatocytes cultured on a floating collagen membrane did not exhibit a typical monolayer formation once the membrane had diminished in size, yet resulted

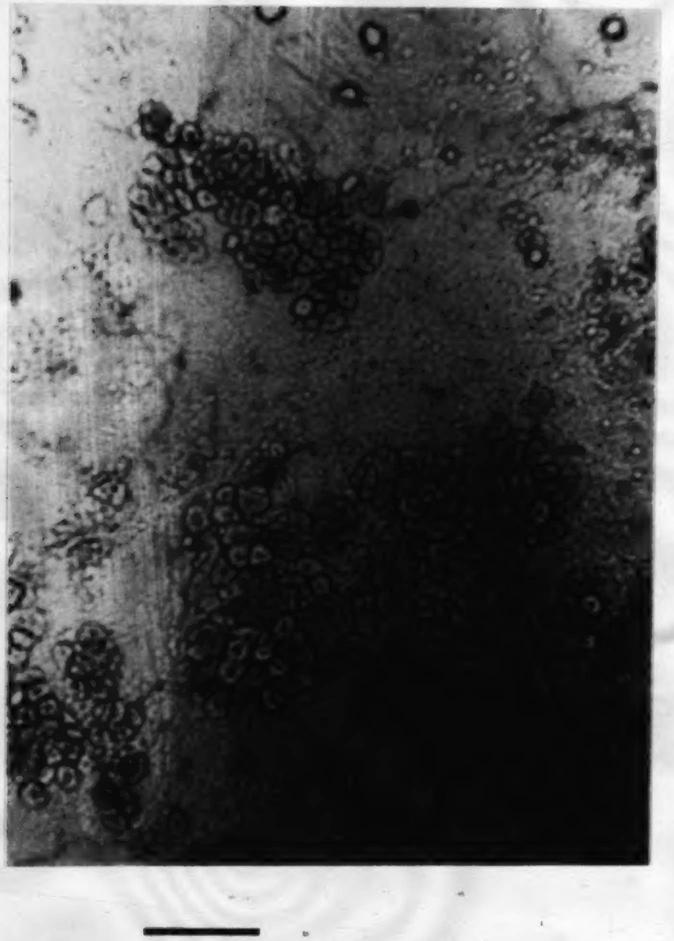
PLATE 8. Primary culture of adult rat hepatocytes plated on a collagen gel (CG) substratum, 96 hours post-inoculation. A) 35x magnification, photographed with angled refracted light; bar = 100 µm. B) 35x magnification, photographed under increased light; bar = 100 µm. Compared to hepatocytes cultured on thin collagen layer (Plate 5), these cells have retained their polygonal morphology and are arranged in smaller clusters.



in the cells maintaining the longest life-span of all substrata tested (Fig. 51). The cells retained a polygonal or square shape which was more characteristic of cells in vivo (Plate 9). Reid et al. (192) recently proposed that a major reason for the lack of success in maintaining functional hepatocytes in vitro was due to the inability to reproduce the same socio-cellular environment (cell-cell relationship) of intact liver tissue. It is possible that the FCM satisfied this requirement by allowing parenchymal cells to anchor themselves to a solid support, while at the same time permitted a certain amount of aggregation to occur. The natural tendency of cells, once dissociated from each other, appears to be for survival by attempting to reconstruct into a morphological arrangement characteristic of their in vivo environment. This may be illustrated further by the rise in LDH activity observed at 3-4 days post-inoculation with hepatocytes cultured on FCM (Fig. 51). It is plausible that because of the improved cell-cell relationship, hepatocytes cultured on the floating membrane can recover more efficiently from the probable trauma sustained during isolation than can cells plated on the other substrata.

Another interesting observation made during the current study was that hepatocytes which failed to adhere securely and flatten out upon a substratum still remained viable for the length of the culture (dye exclusion test) if attached to other cells which had already established into a monolayer

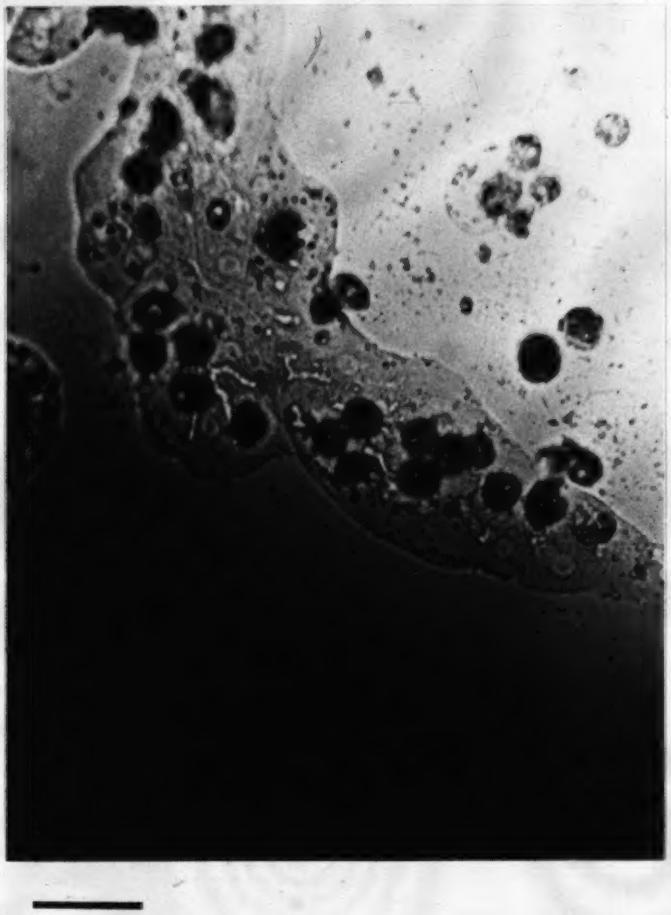
PLATE 9. Photomicrograph of thin slices of intact tissue obtained from adult rat liver (35x magnification). The natural morphology and architecture of cells in hepatic tissue are shown. (Bar = 100 μm)



formation. This occurred despite the fact that these hepatocytes retained a round morphology (Plate 10). In comparison it was noted that cells which displayed the same attachment behavior on the surrounding collagen support, but failed to initiate spreading, rapidly died off. This not only emphasized the importance of proper cell-cell interactions to the survival of the cultures, but questioned further the validity of extensive monolayer formation for maintaining cells in vitro. Why these hepatocytes, plated at the same time as those which established monolayers, delayed attachment until other cells had stablized on a solid support is unclear. It may be, however, a result of more extensive damage sustained during isolation, which reduced their responsiveness towards adhesion to other surfaces. This effect was similar to that observed in studies utilizing cultures of transformed cells as feeder cells for maintaining hepatocytes (see Section 3.3.3.3.1.2). Rat liver parenchymal cells plated on top of established monolayer cultures were shown to increase in both attachment and survival efficiency, with retention of biochemical and morphological features characteristic of cells in vivo, including their polygonal shape.

Recently Reid, Rojkind and coworkers (192, 354), using a specially prepared "biomatrix" as the attachment substratum, were able to maintain functionally viable adult rat liver parenchymal cells for up to six months. This

PLATE 10. Photomicrograph of individual adult rat hepatocytes attached to an established monolayer culture also composed of adult rat liver parenchymal cells (50x magnification). Darker cells represent viable hepatocytes (as determined by trypan blue exclusion) photographed out of the plane of magnification. Microphotograph taken of a 4-5 day culture on a collagen gel substratum. (Bar = 75 μm)



biomatrix, consisting of a complex combination of extracellular matrix components, served to re-establish what Reid et al. referred to as the in vivo socio-cellular variables critical for the survival of differentiated cells in vitro. Hepatocytes plated on this support formed typical monolayer cultures but were able to preserve a polygonal or globular shape for the length of the culture.

Malan-Shibley and Iype (304) noted, with respect to cell shape and architecture, that there were distinct differences between non-proliferating hepatocytes and transformed cells capable of long-term culture. In comparison to non-malignant hepatocytes which were well spread out, thin, and flat when cultured on a solid, rigid support, hepatoma cells grown on the same substratum were more loosely attached and spheroidal in shape. The morphology of these proliferating cells more closely approximated the three dimensional shape of liver cells in vivo than did the flattened out form of the cultured normal liver parenchymal cells. A similar observation was made by Chessebeuf et al. (368) who established long-term cultures utilizing a cell line derived from rat hepatocytes. They noted that cells in the young subcultures, which were unlikely to form into monolayers, had a flat, thin morphology. It was, however, the later subcultures which produced cells which would evolve into polygonal epithelial monolayers. This indicated that it was only when a very viable and rapidly dividing cell line had properly established itself that the cells in culture returned to a polygonal shape.

5.3.3 Determination of Cellular Requirements

In order to ensure that optimal survival time of the non-proliferating cultures, several studies were conducted to determine growth requirements. These studies included a comparison of several media routinely used for maintaining primary hepatocyte cultures, as well as the effect of several components (FBS, insulin, dexamethasone) on cell survival. For this purpose, the testing criteria employed for assessing cellular viability included lactate dehydrogenase (LDH) and tyrosine aminotransferase (TAT) levels and/or dye exclusion.

5.3.3.1 Defined Media

To determine the most proficient medium for maintaining cell survival, primary cultures of non-proliferating adult rat hepatocytes were established on collagen-gel plastic plates, utilizing several commercially available media. Choice of media was based on their use in previously published studies and included L-15 medium (190, 197, 267, 346, 353), Waymouth Medium (MB 752/1) (257, 319, 542), Minimal Essential Medium (MEM) (232, 321, 347), Dulbecco's Modified Essential Medium (DMEM) (187, 279, 282), Ham's F-10 medium (195, 299, 304, 319), Ham's F-12 medium (193, 222, 232, 280, 543, 544), McCoy's 5A (M-5A) medium (291), and

Ham's F-12/DMEM (1/1 ratio) media (545). All cultures were supplemented with 15% FBS, insulin, glucose, and albumin (346). Media were replaced every 48 hours. Viability of cultures was determined by the presence of LDH and/or TAT activity.

These studies demonstrated that the appropriate choice of medium was a critical requirement for mammalian cell survival in culture. Although all media tested were essentially capable of supporting anchorage of freshly isolated hepatocytes to the collagen substratum, media L-15, MEM and M-5A failed to promote monolayer formation to any extent. Of the remaining media in which monolayer cultures did establish themselves, F-12 and DMEM provided the best environment for maintaing viable hepatocytes. Cells cultured in the F-12/DMEM mixture showed a marginally longer survival time period than those in either F-12 or DMEM separately. This response may be due to a type of additive effect in which DMEM supplies the higher concentration of various essential components (292, 546) whereas F-12 provides additional nutritional requirements (547). Subsequently, in all further studies involving isolated hepatocytes, a mixture of DMEM and F-12 was utilized as the basal medium.

5.3.3.2 Fetal Bovine Serum

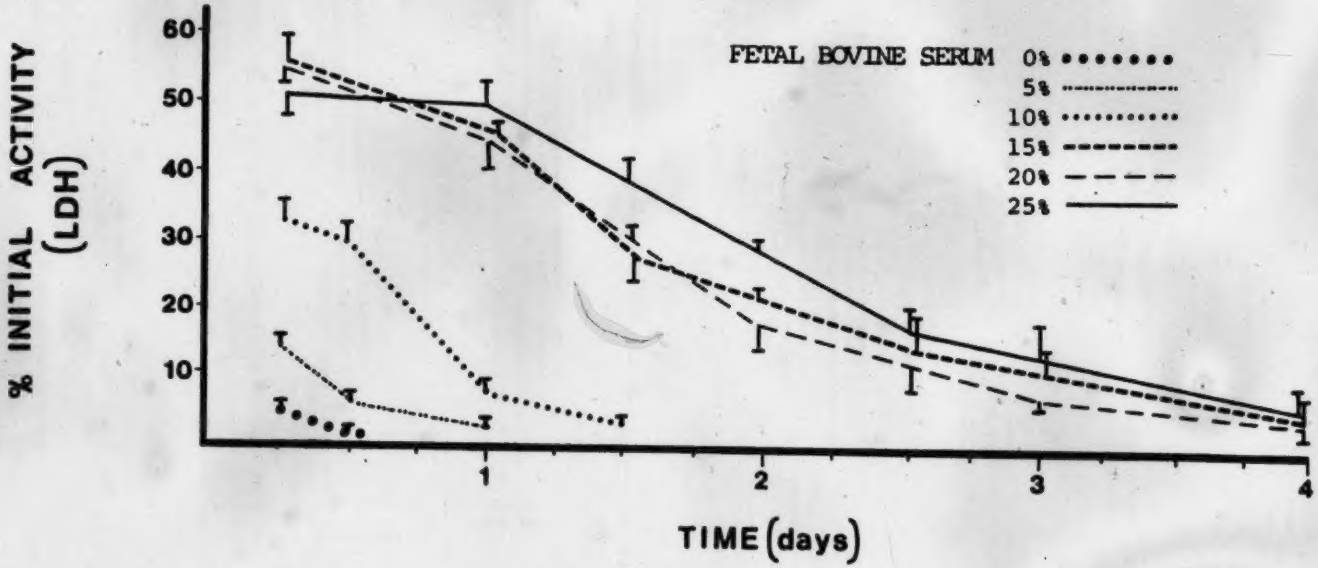
The benefits of using reduced serum or serum-free culture conditions have been discussed elsewhere (287, 291-

293, 548, 549). The advantage in drug metabolism studies is that all components of the culture are essentially known, thus providing a more chemically defined cellular model for hepatic metabolism. Unfortunately, no successes have been reported in maintaining parenchymal cells obtained from normal adult rat liver in the absence of serum, although several investigators have reported that cells derived from partially hepatectomized rat liver could survive up to six days in a serum-free environment (197, 332, 333). Since the addition of serum appeared essential for prolonged cellular viability, it was important to establish the optimum concentration of FBS required for maintaining hepatocytes in primary culture.

Attachment efficiencies of isolated hepatocytes were determined in the presence of increasing levels of fetal bovine serum (0-25%) (Fig. 52). Cells were suspended in F—12/DMEM medium supplemented with insulin, albumin and FBS, and plated on collagen gel petri dishes. Percent attachment of cells was based on LDH activity compared to the freshly isolated cells.

After 6 hours incubation time, the highest percentage of attached hepatocytes (45%) was obtained with a FBS concentration of 15%. Increasing the serum level above 15% did not further increase attachment efficiency. Decreasing the amount of serum added to the medium resulted in a corresponding decline in the number of cells attached, with only about

FIGURE 52. The effect of serum concentration on the attachment efficiency and survival time of adult rat hepatocytes cultured (primary monolayer culture) on collagen gel substratum in the presence of DMEM/F-12 media and insulin. [Determined as a function of the initial lactate dehydrogenase (LDH) activity measured in viable cells] The points represent the means and standard deviations of at least 4 measurements per time period obtained from one or more separate experiments.



5% of the cells capable of anchoring to the substratum in the complete absence of serum. These results agreed with Bonney et al. (198) who reported that adequate levels of fetal bovine serum must be added for proper attachment of the hepatocytes. This was further supported by evidence showing that cold insoluble globulin (CIG), a glycoprotein found in plasma, was found to mediate attachment of cells to collagen (308, 550). In contrast, Rubin et al. (299) could find no evidence to show the presence of serum promoted cell attachment, though they did agree it was necessary for extended survival.

Cultures were also measured for LDH activity at 24 hours post-inoculation (Fig. 52). At depressed serum concentrations (5%, 10%) only very low enzyme levels were noted and no activity was detected in the serum-free system. Because of the low amounts of serum present the small proportion of hepatocytes which were able to attach to the collagen were incapable of establishing a monolayer morphology. This resulted in the rapid deterioration of the cultures. In comparison, with medium supplemented with 15 to 25 percent FBS, monolayers were beginning to form within 24 hours following plating accompanied by only a small decline in LDH activities. Although cultures containing 20 and 25 percent FBS had enzyme levels marginally below that recorded with 15% serum, subsequent measurements demonstrated no difference in survival times of the cells. After four days in culture, approximately one-third of the initial LDH activity still remained, regardless of whether 15, 20 or 25 percent FBS was used.

The above results indicated that sufficient amounts of serum are required for both formation of stable contacts and extended survival of adult hepatocytes. This is in general agreement with most other studies in which levels of FBS between 10 and 25 percent were employed. In all subsequent experiments, 17-20% fetal bovine serum was considered optimum.

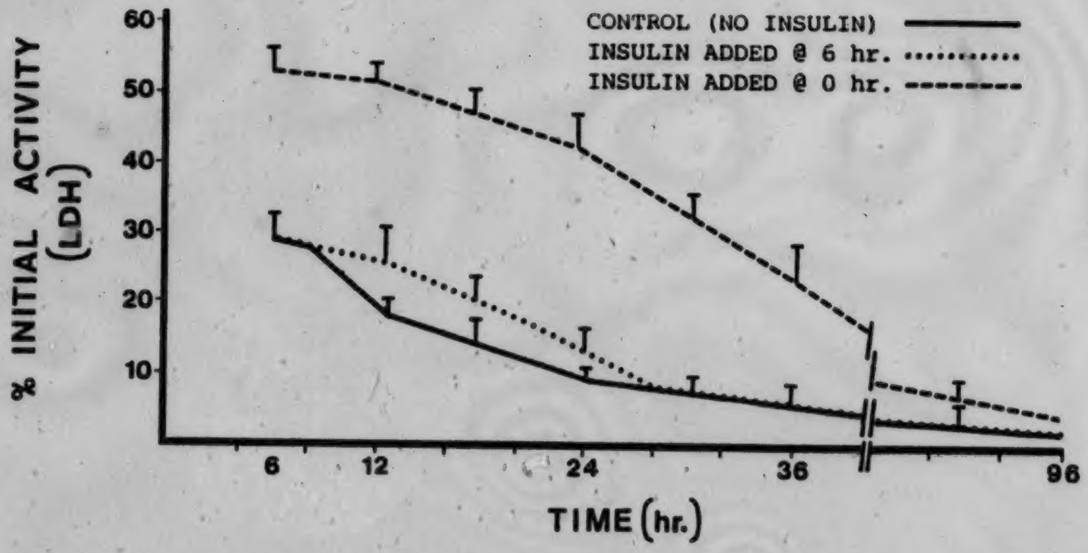
5.3.3.3 Hormonal Supplements: Effect on Cell Suspension and Monolayer Cultures

In a continuation of the study establishing conditions necessary for maximum survival of hepatocytes in vitro, insulin and dexamethasone were investigated as potential growth promoting components. Primary cultures were initiated on collagen gel petri dishes to determine what effect, if any, these hormones had on cell attachment and culture longevity. Furthermore, as an additional indication of their value in prolonging viability, hepatocytes in short - term suspension cultures (< 8 hrs.) were observed for variations in cellular functions which could be attributed to an added presence of insulin and/or dexamethasone.

5.3.3.3.1 Insulin

Although 30% of the hepatocytes seeded in the presence of F-12/DMEM media and FBS were capable of adhering to the collagen substratum, addition of insulin to the culture medium consistently resulted in a higher attachment efficiency (55%) (Fig. 53). Furthermore, the control cultures demonstrated a rapid decline in LDH activity after just 8 hours incubation time, falling to approximately 33% of initial values by 24 hours. In comparison, cultures containing insulin revealed a greater stability, showing only a marginal decrease in LDH activity after 24 hours. Unfortunately, the effect of insulin could not be sustained past this time. A significant loss in enzyme activity was recorded between 24 and 48 hours, and by 96 hours LDH levels could not be differentiated from those of the control cultures. These results substantiated conclusions reached by several investigators who reported that inasmuch as insulin was effective in increasing the attachment efficiency of hepatocytes, it did not extend survival of the cells (198, 288, 335). It was noted, however, that supplementing a control culture with insulin at 6 hours post-inoculation did result in a temporary slowing of the rate of enzyme loss (Fig. 53). This suggested that insulin does exert a direct effect on certain cellular functions, but whether the result is morphological or biochemical is unclear.

FIGURE 53. The effect of insulin on the survival time of adult rat hepatocytes plated in monolayer culture (upon collagen gel substratum) in the presence of DMEM/F-12 media and FBS. [Determined as a function of the initial lactate dehydrogenase (LDH) activity measured in viable cells] The points represent the means and standard deviations of at least 6 measurements per time period obtained from at least 3 separate experiments.



In contrast to the greater stability displayed by monolayer cultures in the presence of insulin (Fig. 53), the addition of insulin to hepatocyte suspension cultures resulted in no apparent improvement in cell viability (Fig. 54). The rate of decline in the number of cells capable of excluding trypan blue, as well as the decreases in LDH and TAT activities paralleled those observed with the control cultures (absence of insulin). It was apparent that any possible effects resulting from the action of insulin were too slow in materializing to decrease the rapid deterioration of hepatocytes which typically occurs in suspension.

5.3.3.3.2 Dexamethasone

Several investigators have reported that the addition of corticosteroids to a culture medium provides some essential requirement, which results in a more favorable environment for maintaining non-proliferating cultures (335, 542). In the present study, however, the inclusion of dexamethasone resulted in no measurable improvement in the properties of hepatocytes initiated as primary monolayer cultures (Fig. 55). Neither an increase in the attachment efficiency of the cells, nor an extension of the survival rate was noted when compared to a control culture (absence of dexamethasone). Still, this observation may be a reflection of the choice of the parameter measured (LDH activity) rather

FIGURE 54. The effects of insulin and/or dexamethasone on the viability of adult rat hepatocytes in suspension culture (in the presence of DMEM/F-12 media and FBS). Effects were determined as measurable changes in certain viability markers: A) trypan blue exclusion test; B) level of lactate dehydrogenase (LDH) activity; C) level of tyrosine aminotransferase (TAT) activity. The points represent the means and standard deviations of at least 5 measurements per time period obtained from at least 3 separate experiments.

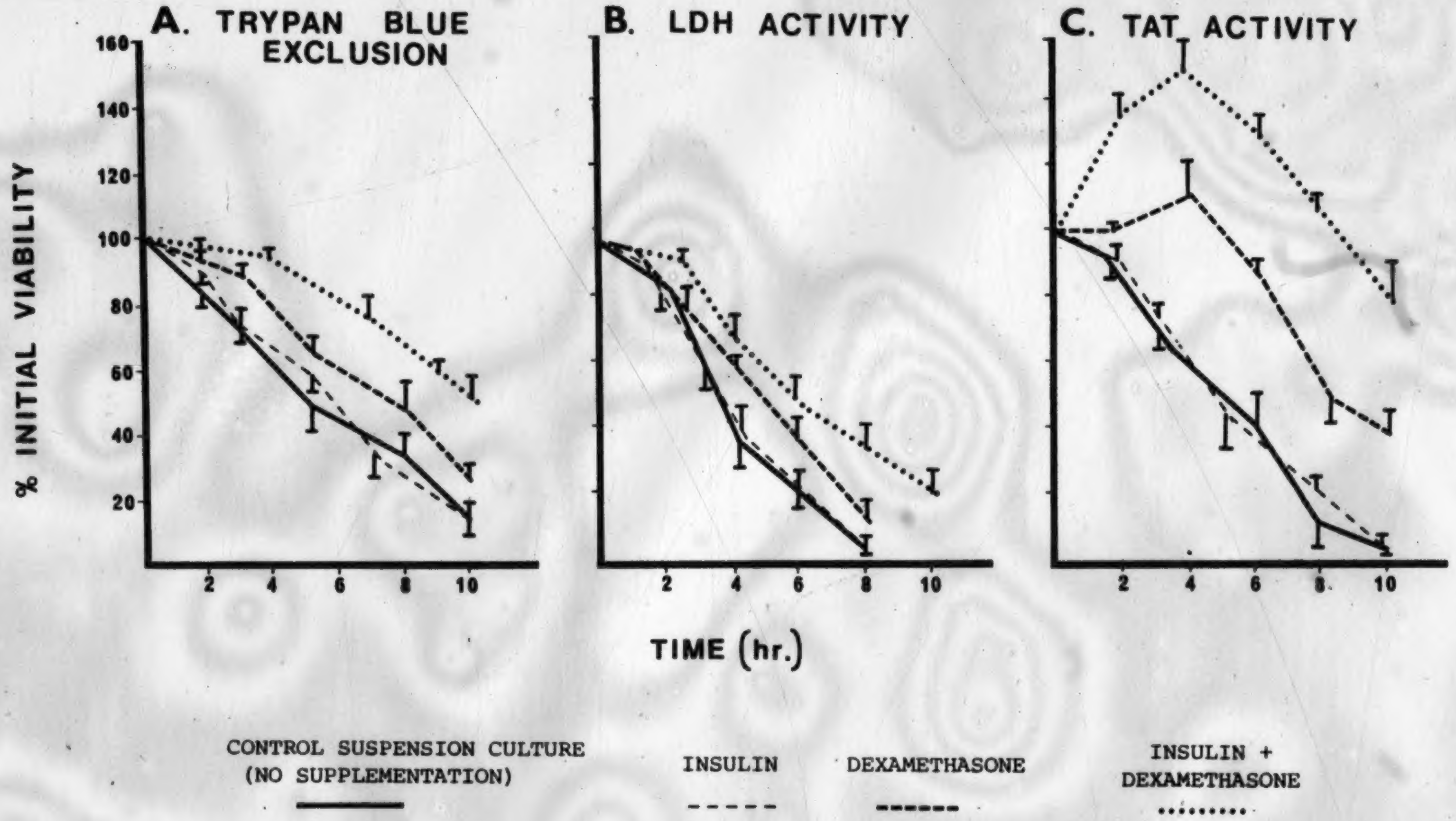
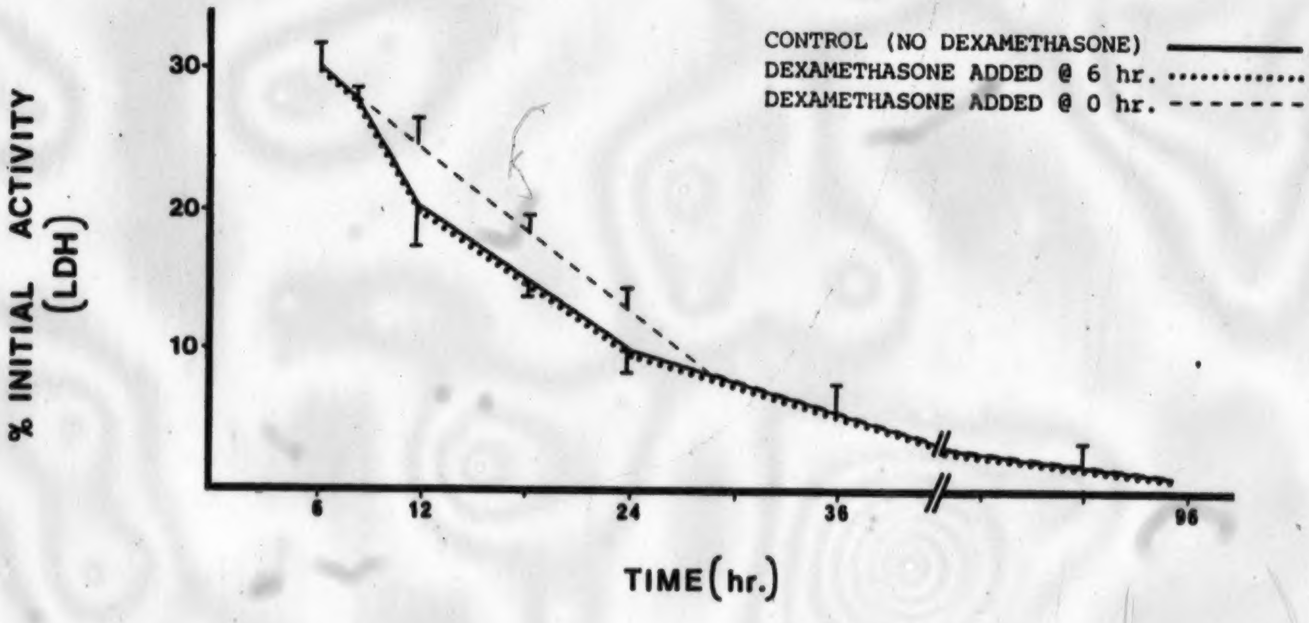


FIGURE 55. The effect of dexamethasone on the survival time of adult rat hepatocytes plated in monolayer culture (upon collagen gel substratum) in the presence of DMEM/F-12 media and FBS. [Determined as a function of the initial lactate dehydrogenase (LDH) activity meassured in viable cells] The points represent the means and standard deviations of at least 6 measurements per time period obtained from at least 3 experiments.



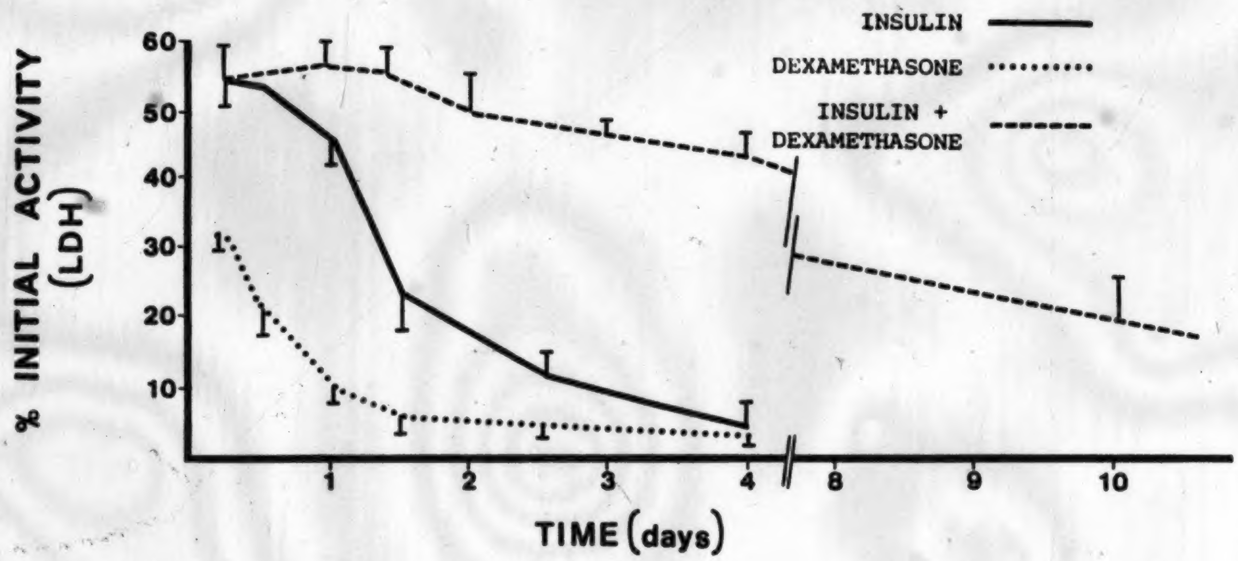
than due to any complete lack of response. It was noted that the addition of dexamethasone to short-term suspension cultures did induce certain changes in the cells, although LDH activity was not one of them (Fig. 54B). Based on dye uptake, the presence of dexamethasone consistently appeared to improve structural stability, as the rate at which cell integrity was lost was marginally slowed (Fig. 54A). Laishes and Williams (335) considered dexamethasone's action on cell surface membranes as the most likely basis for its enhancement of survival. Similarily, Freidman and Epstein (238) stated that glucocorticoids prevented enzyme loss by improving structural integrity, as well as promoting synthesis of various enzymes. This latter effect was demonstrated both in the present study (Fig. 54B) and in others (190, 198, 232, 343, 346, 551) by the induction of TAT activity in suspension culture. Although corticosteriods can directly influence certain liver enzymes such as TAT (198, 343), numerous other enzymes are not affected. Illustrative of this are LDH levels of suspended cells which were raised only marginally in the presence of dexamethasone. However, as this observed increase paralleled the improvement also seen in the dye exclusion test upon addition of dexamethasone (Fig. 54A), the higher level of LDH may only reflect the hormone's influence on cellular integrity and the prevention of enzyme leakage, rather than any effect on the enzyme directly.

5.3.3.3.3 Combined Hormonal Addition

Despite the marginal successes in the enhancement of the survival of hepatocytes in vitro upon the addition of either insulin or dexamethasone, it was discovered that significant improvements in cell viability did occur when both these components were added simultaneously to the culture medium (Fig. 56). Although this concurrent supplementation did not increase attachment efficiency above that attained with insulin alone, survival times of primary monolayer cultures were substantially extended. In the presence of both hormones, viable cells were maintained for over 14 days. In some instances, 20% of the initial LDH activity was still detected after 10 days in culture. In comparison, earlier attempts to culture hepatocytes resulted in the majority of cells dying within 4 to 5 days.

The dramatic increase in survival time generated by the combined action of both hormones was contradictory to findings obtained by Laishes and Williams (335). They reported that improvements in cellular functions brought about by dexamethasone, were in fact, inhibited by the simultaneous presence of insulin, and concluded that because glucocorticosteriods and insulin have opposing effects as far as liver functions are concerned (i.e. dexamethasone stimulates gluconeogenesis and glycogen storage, while insulin promotes glycolysis), insulin has an antagonistic influence on the ability of dexamethasone to increase cell longevity.

FIGURE 56. The effect of the concurrent addition of insulin and dexamethasone on the viability of adult rat hepatocytes plated in monolayer culture (upon collagen gel substratum), in the presence of DMEM/F-12 media and FBS. [Determined as a function of the initial lactate dehydrogenase (LDH) activity measured in viable cells] The points represent the means and standard deviations of at least 6 measurements per time period obtained from at least 2 separate experiments.

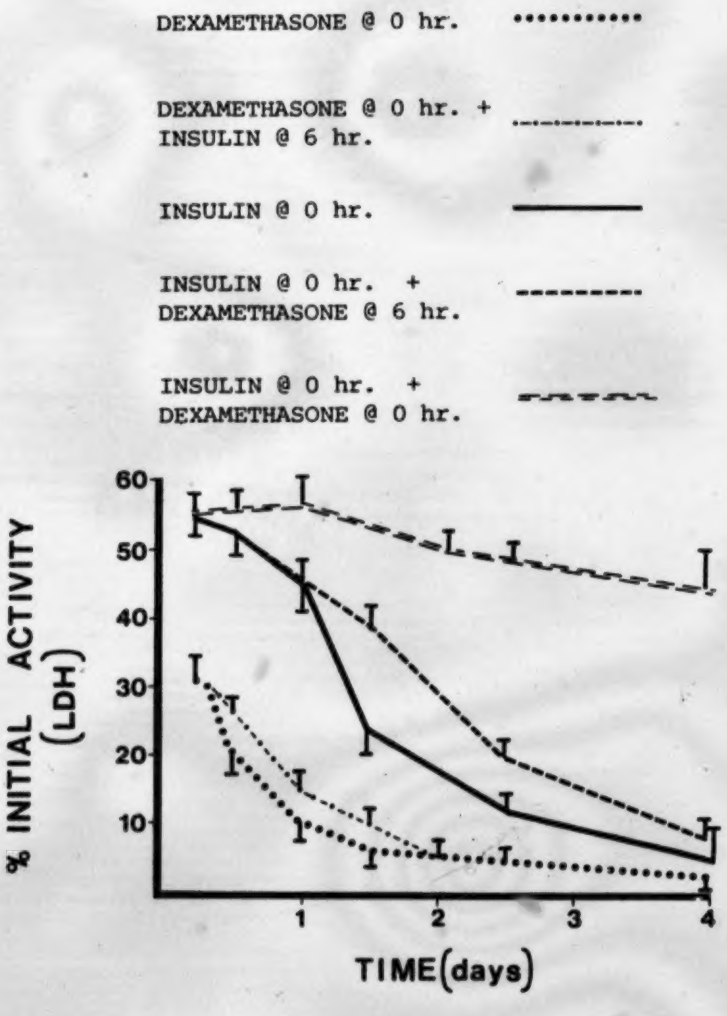


In support of the current results though, Bonney et al. (198) were able to demonstrate that the presence of both insulin and dexamethasone resulted in an increased synthesis of several enzymes by cultured hepatocytes.

The value of using a combination of insulin and dexamethasone was indicated further by the results shown in Figure 57. In this study addition of the dexamethasone to culture medium already containing insulin was delayed until 6 hours post-inoculation, at which time medium and non-attached cells were replaced with fresh medium containing both hormones. In this way, it was possible to demonstrate that dexamethasone had a direct and rapid effect on sustaining cell viability when insulin was also present. Bonney et al. (198) similarly found that the addition of dexamethasone to insulin-supplemented medium resulted in immediate higher enzyme levels. In comparison, Yamada et al. (343) reported that the effects of dexamethasone required at least six hours before any variation from the controls were noted.

When the addition sequence of the two compounds was reversed, that is, with dexamethasone present initially and the addition of insulin delayed, only a moderate increase in LDH activity occurred. The results obtained in the experiment just described suggested that the function of dexamethasone was more important later in culture, after cells had attached themselves securely to a solid substratum, and added support to the contention that a major role of insulin is to

FIGURE 57. The effects of delayed addition of insulin and/or dexamethasone on the survival time of adult rat hepatocytes which have already established their initial anchorage (upon a collagen gel substratum) towards forming a monolayer culture. [Determined as a function of the initial lactate dehydrogenase (LDH) activity measured in viable cells] The points represent the means and standard deviations of at least 6 measurements per time period obtained from at least 2 separate experiments.



aid the formation of these stable contacts. Regardless of their exact function, it was clear that the presence of both hormones at time zero prolonged substantially the survival period of hepatocytes *in vitro* compared to cultures in which the addition of either hormone was delayed.

Improvements similar to those seen in monolayer culture were noted with hepatocytes suspended in medium containing both insulin and dexamethasone. Using short-term hepatocyte suspension cultures, the present study demonstrated that, when supplemented together these hormones significantly enhanced the specific activity of cellular TAT as well as delayed the rate at which structural deterioration of these cells occurred (Figures 54A and 54C). LDH activity, however, was only marginally increased (Fig. 54B). In the presence of both hormones, TAT activity after 4 hours was increased to approximately 150% that of basal enzyme levels (Fig. 54C). In direct comparison, after the same time period, cell suspensions supplemented with only the dexamethasone achieved levels of 110%, whereas control values had dropped to 60-70% of the initial level. The combination of insulin and dexamethasone was also superior in maintaining the cellular integrity of hepatocytes in suspension. In Figure 54A, it can be seen that the rate of cell loss was slower than that measured in the control, or in suspension cultures supplemented solely with either insulin or dexamethasone.

Despite the initial improvements in cellular viability obtained, the effects produced by simultaneous addition of dexamethasone and insulin were not sustained in suspension culture, even with subsequent inclusions of both hormones. After 4 hours incubation time, TAT levels decreased at a rate which was comparable to that observed with the control suspensions. A similar decline after about 4 hours was noted in the population of cells capable of excluding trypan blue. Using both TAT levels and dye exclusion as viability markers, the overall result of augmenting hepatocyte suspensions with both insulin and dexamethasone was to increase the functional life-span of suspension cultures from approximately 8 hours to 14-15 hours. It is impossible to explain, however, why insulin and dexamethasone together increased cellular survival but separately had little or no effect on cell viability. Apparently neither hormone alone can be a single controlling factor, but together play a co-operative role in the regulation of cell functions.

5.3.4 Fetal and Neonatal Rat Liver Cells

5.3.4.1 Isolation of Cells

The procedure for the isolation of fetal and neonatal rat hepatocytes was adapted from the methods of Kaighn (552) and Acosta _et al_. (282). Livers were excised from immature rats (15 day _p.c._ to 10 day neonatal) and subjected to a series of enzyme-mediated dissociation steps as outlined in

Section 4.3.3.2.2. A major modification was made in the reported dissociation medium. In the present study, two enzymes in combination were employed, viz. 0.05% trypsin in PBS, and 0.05% collagenase buffer (71). Hepatic tissues were incubated with reciprocating shaking for 20 minutes at 37°C in 10 volumes of the dissociation medium (trypsin/collagenase ratio 1/5). The supernatant from the first enzymic treatment contained mainly blood cells and some tissue debris and was discarded. Three further enzymic incubations were carried out using successive trypsin/collagenase ratios and incubation times of 2/5 , 20 min. ; 1/1 , 15 min.; and 5/2 , 10 min. The combined supernatants from these incubations were filtered to remove undigested tissue. Collection of the dispersed liver cells was subsequently achieved by low speed centrifugation.

The rationale for incubating the hepatic tissue with a series of collagenase buffers containing increasing proportions of trypsin was to reduce any unnecessary damage to the cells caused by enzymic treatment. Either trypsin or collagenase are commonly employed to disperse fetal liver tissue into single cells and although trypsin has been the traditional choice, no obvious advantage to using one enzyme instead of the other has been demonstrated. However, several studies have indicated that prolonged exposure of hepatocytes to trypsin may result in damage occurring to the cellular membrane (290, 304, 326). In contrast and for reasons which are unclear, Gallai-Hatchard and Gray (553) were able to show that utilization of a combination of collagenase and trypsin yielded cells of a better viability than was possible using either enzyme alone. Other investigators (328, 375, 378, 552) have confirmed the observation of Gallai-Hatchard and Gray. The same combination of enzymes was used in the present study.

Dissociation of livers obtained from embryonic or post-natal rats yielded approximately 32 x 10^6 cells per g wet tissue, with a viability index routinely between 65 and 75 percent. This was equivalent to a viable cell population of about 22.4 x 10^6 hepatocytes/g liver, or 4.1% of the total possible yield (554). These cell yields proved favorable as the maximum possible recovery of isolated hepatocytes obtained through enzymic digestion of liver fragments has been only about 5% of the total number of parenchymal cells (5, 326).

Examination of the recovered cells by low magnification microscopy revealed a mixture of two cell types, parenchymal hepatocytes and fibroblast-like cells. The younger the liver, however, the more difficult it was to distinguish these cells morphologically. Hepatocytes occurred primarily as single cells, although some small cell aggregates composed of 2-5 hepatocytes were also present. No tetraploid hepatocytes were observed. Attempts to purify the hepatocyte suspension by differential centrifugation were unsuccessful.

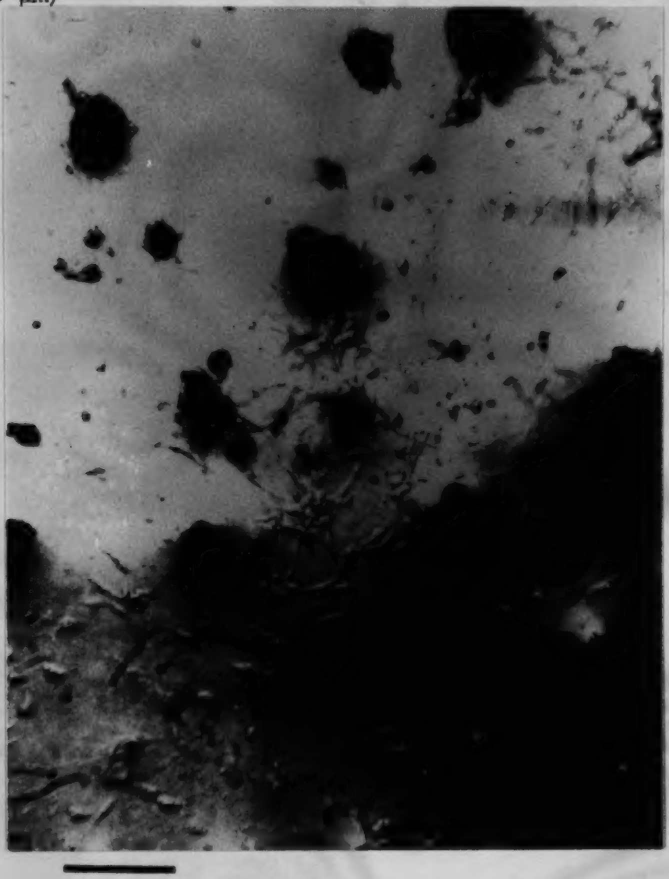
5.3.4.2 Monolayer Cultures

Isolated fetal liver cells were suspended in F-12/DMEM media supplemented with 20% FBS, insulin, dexamethasone and antibiotics, and approximately 3 x 10^6 cells in 3 ml medium were plated onto a collagen gel substratum. Plating efficiency easily reached levels of 70-80%. However, the attachment efficiency of these cells varied (50-80%) from experiment to experiment to a greater degree than that observed with adult hepatocytes (50-60%). This may be due to the tendency of a significant number of the dissociated immature cells to aggregate into clusters, which subsequently interfered with their ability to adhere properly to the support. Despite this clumping, the overall behavior of these cells during the first days in culture was similar to that of cultured adult hepatocytes.

5.3.4.2.1 Purification of Endothelial Cell Cultures

The major difficulty encountered with primary cultures of immature rat hepatocytes was contamination of the cultures with non-parenchymal cells. Although fibroblastic cells appeared few in number for the first 2-3 days, they proliferated actively during the ensuing days. Identified by their stellate shape in cultures more than one day old (Plate 11), these cells flattened out and spread rapidly in all directions. By days 4-5 fibroblast-like cells dominated

PLATE 11. Primary culture of fetal rat liver cells seeded on collagen coated plates, 2 days post-inoculation (35x magnification). At this time the presence of a large number of fibroblastoid-like cells (stellate-shaped) are already observed. Darker areas represent aggregates of fetal cells which are out of the plane of the microscope. (Bar = 100 µm)



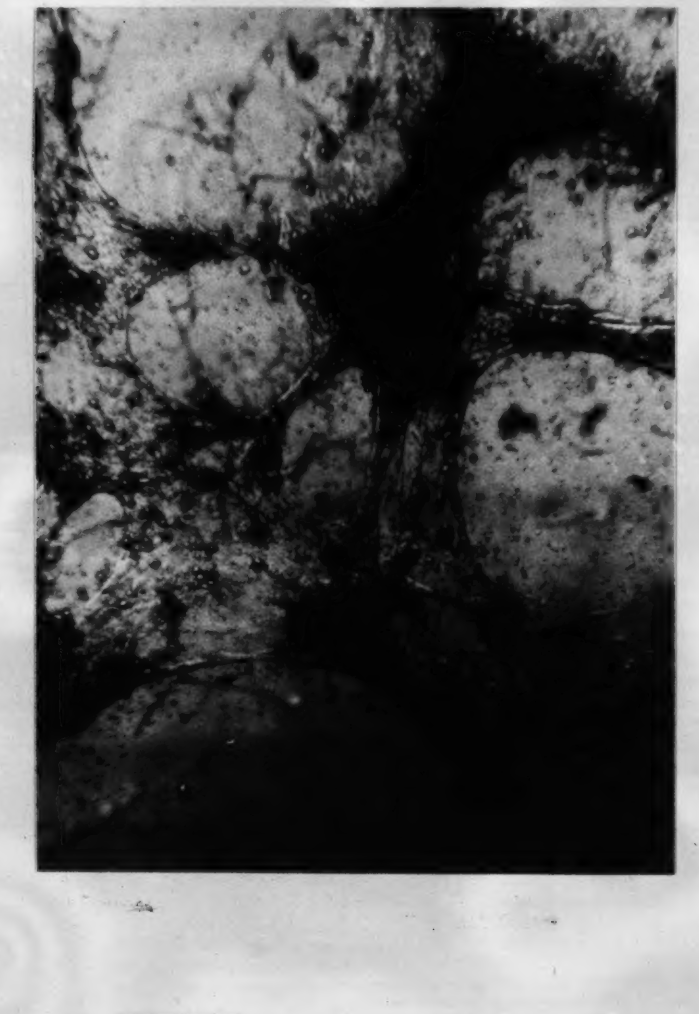
the cell monolayer and eventually overgrew the epithelial cells completely (Plate 12).

Several studies have demonstrated that the contamination problem could be avoided. Acosta et al. (343) and Leffert and Paul (279) were able to inhibit fibroblast overgrowth by growing the cultures in a selective arginine-deficient medium. William et al. (321) and Takaoka et al. (284) however, relied on differential attachment to a culture surface to separate the hepatocytes from the fibroblast-like cells.

In the present study attempts were made to separate the two cell populations in a manner similar to that described by Tsiquaye et al. (274). Cell suspensions containing the two cell types were seeded onto plastic petri dishes and incubated at 37° C for 2 hours. During this period fibroblast cells attached more rapidly, leaving a purer suspension of parenchymal cells to be recovered. This suspension was replated sequentially upon plastic until it appeared that only the parenchymal cells remained. Unfortunately, because the rat embryonic hepatocytes also demonstrated a willingness to attach to tissue culture plastic, a significant portion (up to 70%) of the viable liver parenchymal cell population was lost during this purification procedure.

Using the purified suspension, primary cultures containing predominantly hepatocytes were set up on collagen gel

PLATE 12. Primary culture of fetal rat liver cells showing overgrowth of the parenchymal cell culture by fibroblastic cells. Microphotograph taken of a 4-5 day culture. (Bar = 500 µm)



plates. Many of the cells aggregated into small clumps, but this did not interfere with the general behavior of the culture. After several hours incubation, the majority of hepatocytes had firmly attached to the substratum and established contact with each other (Plate 13). No fibroblastoid cells were apparent. Islands of these compact hepatocytes quickly spread out into closely adhering sheet-like monolayers. Establishment of monolayer morphology and loss of individual polygonal cell shape occurred much earlier (within 1-2 days) in cultures of immature parenchymal cells than with adult hepatocytes (4-5 days). Immature cell cultures maintained an epithelial morphology for 10 to 12 days. Increasing number of fibroblastic cells, which appeared after the first week in culture, began to dominate at this time. Cultures were typically fibroblastoid within two weeks after plating. Absolute elimination of fibroblasts was never successful using differential attachment techniques. In retrospect, however, this should not have been totally unexpected. In order for Williams et al. (321) to obtain a pure parenchymal culture, sequential passage of the cell suspension onto plastic was neccesary until only about 2% of the starting pool of cells was left for culturing. Treatment of their crude cell suspension was considerably more proficient in removing the fibroblastic cells than in the current study where 30-40% of the cells remained, however, their final 2% represented less than 10^4

PLATE 13. Fetal rat liver cells seeded on collagen coated plates, 4 hours post-inoculation (35x magnification). Liver parenchymal cells from fetal tissue have established into monolayer formation after this short time, compared to 24-48 hours necessary for adult liver parenchymal cells (Plate 5).
(Bar = 100 µm)

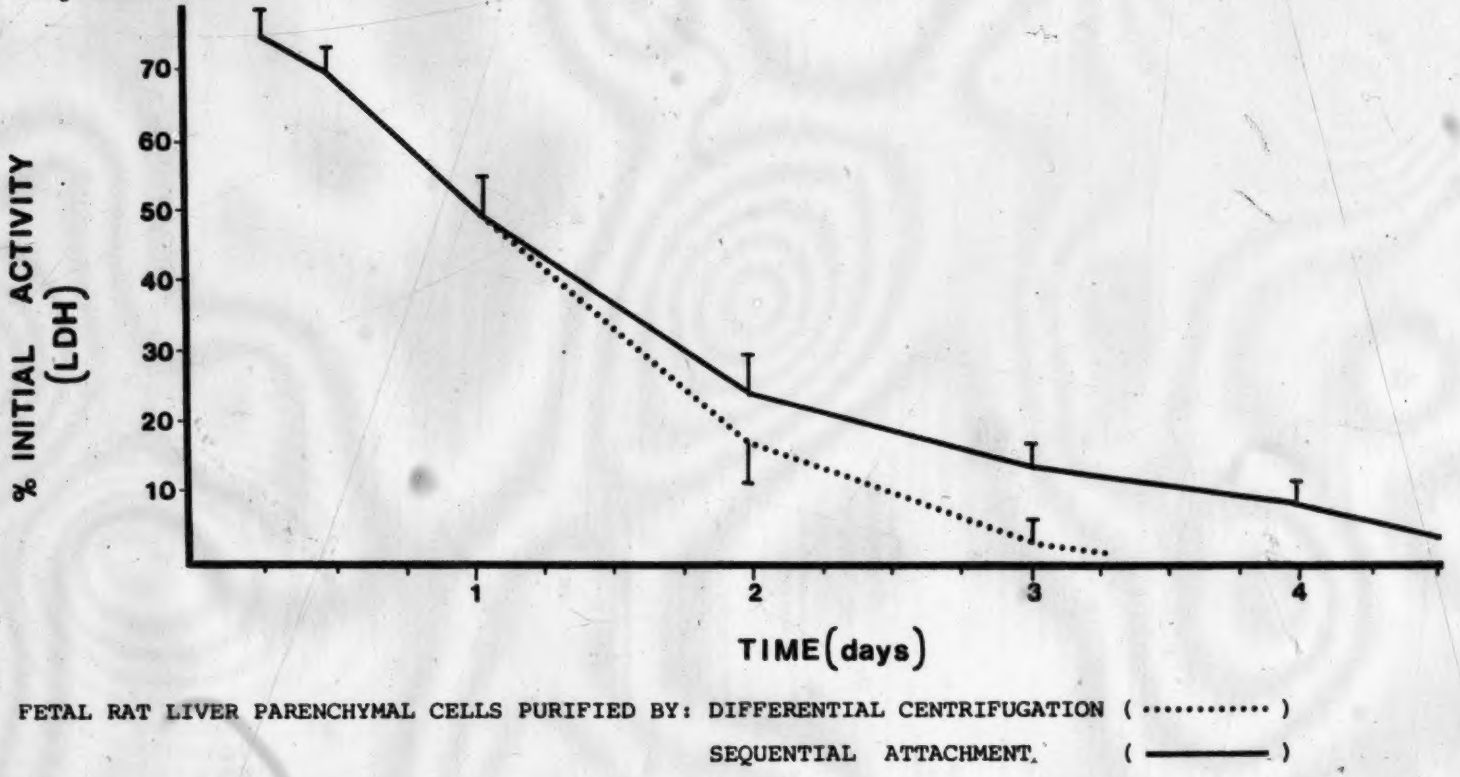
hepatocytes to work with.

5.3.4.2.2 Viability of Functional Cultures

In spite of the moderate success in extending the viable life-span of fetal hepatocyte cultures by delaying fibroblastic overgrowth, there was essentially no difference in enzyme activity (LDH) between any of the cultures established (Fig. 58). Fetal rat liver parenchymal cells only weakly expressed hepatocyte-like functions in vitro, and, unlike adult hepatocytes where the status of culture could be accurately monitored by enzyme activities, it was questionable whether similiar studies faithfully reflected the growth pattern of fetal cells in culture. LDH levels began showing a rapid decline just 12 hours after plating the fetal hepatocytes and was usually completely lost by 4-5 days in culture, despite the obviously prolific nature of the fetal cultures. The slightly faster fall in the LDH level seen with cultures from the non-purified hepatocytes as compared to that of the purified cell suspension, was attributed to the earlier appearance of the fibroblastic cells.

Why fetal rat liver epithelial cells are incapable of maintaining specific hepatic functions in vitro is not completely understood, however, it appears to be a problem shared with that of adult hepatocytes. The presence of non-parenchymal cells, although troublesome for long-term

FIGURE 58. A comparison of the survival times of fetal rat liver parenchymal cells in primary monolayer culture following purification of hepatocytes by either differential centrifugation or sequential attachment. [Cells were plated on collagen gel in the presence of insulin, dexamethasone, FBS and DMEM/F-12 media, and viability of the hepatocyte cultures was determined by monitoring enzymic activity (lactate dehydrogenase, LDH) characteristic for parenchymal cells] The points represent the means and standard deviations of at least 7 measurements per time period obtained from at least 2 separate experiments.



cultures, is not the reason since cultures established from the purified hepatocyte suspensions lost all LDH activity before fibroblastic cells became evident. Tsiquaye et al. (274) have suggested that loss of cellular characteristics may be the result of morphological alterations to the normal fetal hepatocytes in response to the culture conditions. This was evident in the current study where the rapid decline of enzyme activity correlated well with the rapidity and extent to which fetal hepatocytes in monolayer formation lost their characteristic polygonal shape. These functional limitations are probably more than just a result of unstable cell contacts, however, and probably also reflect an inadequacy in the nutritional environment which may prevent immature hepatocytes from differentiating into fully mature cells in vitro (5, 281, 304, 374, 555, 556). Several investigators have established long-term cultures from fetal or neonatal hepatocytes, but the resulting cultures possessed few of the definitive hepatic-like properties of mature hepatocytes (221, 222, 227-229, 321, 361, 365, 368-374). There is increasing evidence to indicate that the precursors of any proliferating endothelial cultures (adult or embryonic) are actually hepatic stem cells (323, 374, 380). These incompletely differentiated hepatic cells account for the few weak hepatocytic-like functions expressed by these long-term cultures, but do not represent true liver parenchymal cells.

5.3.4.3 Suspension Cultures

Isolated embryonic rat liver cells, which were not segregated by differential attachment, were initiated in suspension culture. It was observed that under certain conditions dissociated cells could be induced to aggregate into compact clumps, which subsequently re-established a tissue-like continuity. When examined under the microscope, the resultant pseudo-tissue bore a resemblance to liver tissue, being comprised primarily of parenchymal and connective cells (Plate 14).

The process of embryonic cells re-organizing _in vitro_ into multicellular systems is a common, but still inadequately understood, observation (268, 557-560). Under appropriate conditions, primary cells give rise to typically differentiated structures in accordance with the architecture and character of the original tissue. The reasons for this phenomenon are many and complex, but basically represent the characteristic behavior of immature cells during morphogenesis, differentiation, growth, or regeneration (268, 559, 561). It has been suggested that this process is largely dependent on the presence of, and interaction with, specific cell-surface components which function in cell recognition and cell affinities. This enables cells to aggregate morphogenetically and associate selectively into tissue

PLATE 14. Section of a 72 hour aggregate of fetal rat liver cells, showing formation of a pseudo-tissue which occurs with fetal cells in suspension culture (35x magnification). Cells were cultured as described in Materials and Methods. Aggregate represents a point in time of peak intercellular adhesiveness; explanation in text. (Bar = 100 µm)

patterns (560-563). The ability of cell suspensions to form aggregates is highly age- and tissue-specific.

It was demonstrated in the present study that by using low-speed (< 60 rpm) rotation-mediated cell aggregation, it was possible to reconstruct multicellular structures from embryonic liver cells. After 2 hours of rotation in flasks, dissociated cells had formed into small clusters. After 6 hours in suspension, the clusters had become larger, although a significant portion of the suspension was still single cells. By 24 hours all of the cells appeared to have aggregated into clumps, but those cells incapable of aggregating while in suspension probably deteriorated during this period of time, leaving the impression that few single cells remained. Within 48-72 hours the aggregated cells had formed a large membrane-type structure, 2-5 cells thick. Its appearance was very similar to that observed with adult hepatocytes cultured on a floating collagen membrane. Liver parenchymal cells were far less abundant than connective cells, although specific areas of the membrane were established where close contacts between hepatocytes were formed and the cells retained a spheroidal morphology. Connective tissue, made up essentially of cells lacking any real definitive morphology, comprised the bulk of the membrane and appeared to form a basic support for the parenchymal cells.

Unfortunately the inconsistency of aggregate formation made continued studies on this topic difficult. Standardized results could not be achieved. On many occasions throughout the study the embryonic cells failed to re-organize or simply aggregated into small fragmented membranes. Moscona (268, 564) pointed out that aggregation of isolated cells in vitro was greatly dependent on the culture conditions and cells rapidly lost their capacity to re-associate. Because of this, further investigations on the survival of fetal hepatocytes as pseudo-tissues were not continued. The limited findings just described, however, support the contention (192, 354) that dissociated cells will attempt to reconstruct and preserve a proper socio-cellular environment in order to enhance survival. Cells which did not demonstrate an affinity for each other rapidly died off when in suspension.

5.3.5 Immobilized Hepatocytes

At present, the immobilization of viable microbial cells has attracted a considerable amount of interest and many studies on the subject have been published (see reviews 565-568). Of particular interest has been the observation that immobilized cells are much more stable than cells freely suspended in liquid (569, 570). In spite of the obviously important implications of this finding, few studies on the immobilization of viable mammalian cells have been initiated.

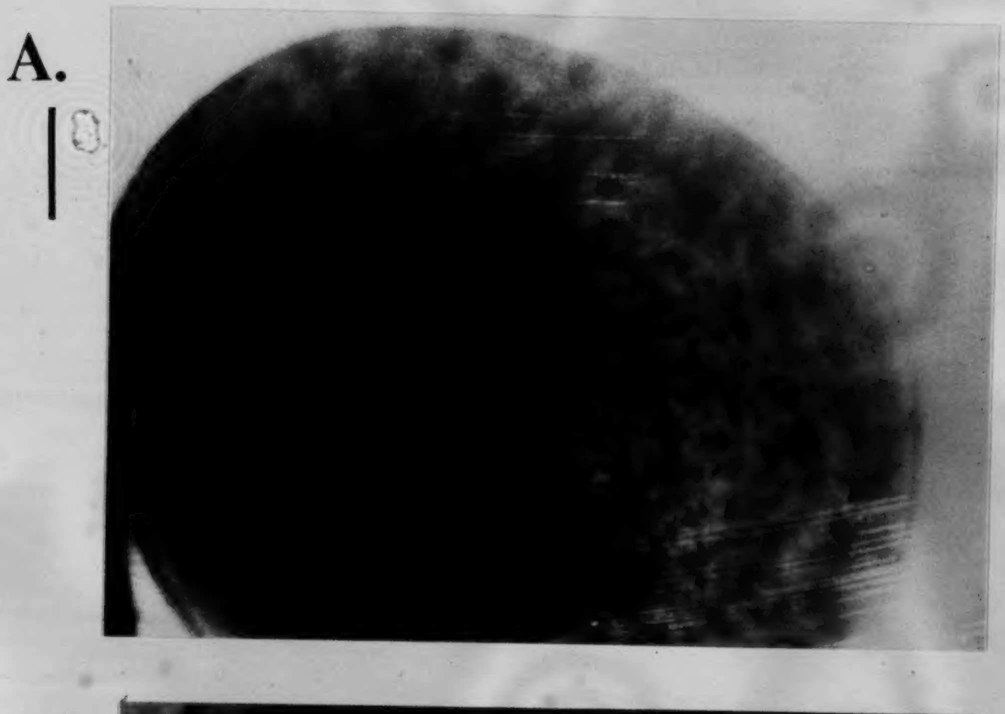
The present study reports on the immobilization of adult rat hepatocytes and its effect on the survival of these cells.

5.3.5.1 Immobilization of Adult Rat Hepatocytes

Conventional immobilization techniques usually involve preparative conditions which could cause cellular damage; for example (i) contact of the aqueous phase, where the cells are located, with an organic solvent or, (ii) elevated temperatures. Both situations must be avoided if mammalian cells are to remain viable. In the current study a process employed for the immobilization of microbial cells (and enzymes) was adapted for use with the much more fragile mammalian cells (571).

Essentially, freshly isolated adult rat hepatocytes were suspended in a prewarmed (40°C) aqueous solution of 5% carrageenan. Small droplets of this suspension were rapidly gelled by immersing into an isotonic Tris-KCl-$CaCl_2$ buffer maintained at room temperature. The resulting immobilized preparations were stable, semi-permeable, bead-type gels, about 3mm in diameter (Plate 15). The size of beads was dependent on the rate of addition of the droplets to the gel-inducing buffer solution. The final sphere size was the product of a compromise between speed of preparation and desired bead size. Because this method involved an all-aqueous phase system, and temperatures used were only marginally elevated for a very short time, it was possible to

PLATE 15. Photomicrograph of adult rat hepatocytes entrapped within a a semi-permeable carrageenan matrix bead.
A) 12x magnification (Bar = 500 µm); B) 35x magnification (Bar = 100 µm).



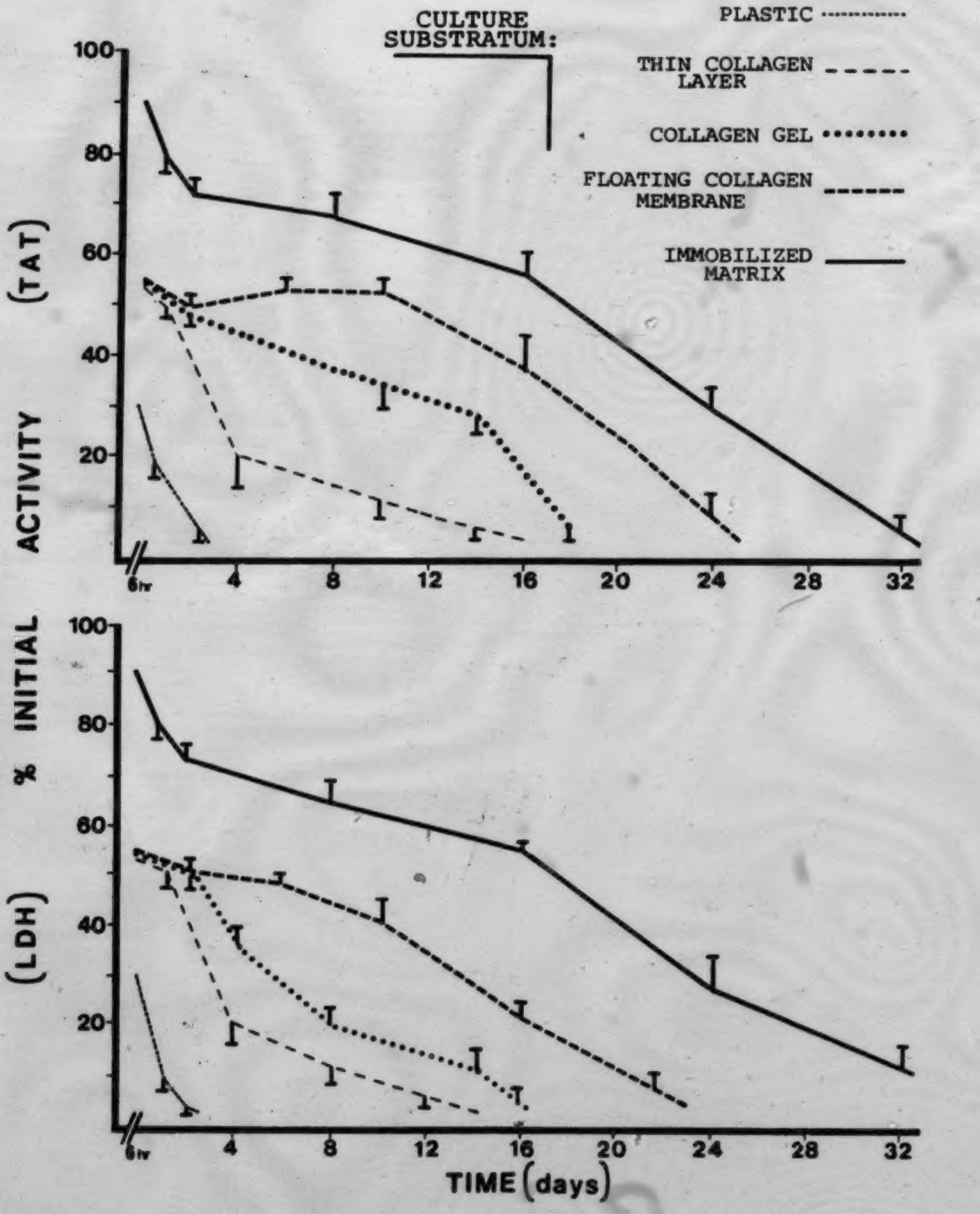
eliminate the above mentioned problems. Cell entrapped carrageenan beads were incubated at 37°C in tissue culture plates containing F-12/DMEM/KCl + $CaCl_2$ media supplemented with 20% FBS, insulin, and dexamethasone. Viability and growth pattern of the entrapped cells were monitored by enzyme activity (LDH) and light microscopy.

5.3.5.2 Viability of Immobilized Hepatocytes

The measured enzyme levels, illustrated in Figure 59, demonstrated that hepatocytes contained in this immobilized system could be maintained in a viable state for between 4 to 5 weeks. This was substantially longer than hepatocytes cultured on more conventional supports (plastic, 2d; thin collagen layer, 14d; collagen gel, 16d; floating collagen membrane, 23d). In related studies, Lim and Moss (572) also observed a marked enhancement in the survival times of rat hepatomas and pancreatic islets following entrapment in sodium alginate beads, and Pilwat et al. (573) were able to significantly prolong the storage period of human red blood cells by immobilizing the cells in a similar alginate matrix.

The currently obtained carrageenan beads were examined under a light microscope and the embedded hepatocytes were found to have retained their spheroidal shape. There were no significant changes in the cell morphology during the period of entrapment. Termination of cultures was determined by the complete lack of enzyme activities (LDH, TAT). Carrageenan

FIGURE 59. The effect on the survival time of adult rat hepatocytes by immobilization of the cells within a semi-permeable carrageenan matrix. [Cells were maintained in the presence of insulin, dexamethasone, and DMEM/F-12/KCl + $CaCl_2$ media plus FBS, and viability based on lactate dehydrogenase (LDH) and tyrosine aminotransferase (TAT) activities measured in viable cells] The points represent the means and standard deviations of at least 5 measurements per time period obtained from at least 2 separate experiments.



beads remained essentially stable for the active period of the cultures, although after four weeks incubation time, signs of disintegration were becoming evident.

Carrageenan is used for the immobilization of enzymes as well as cells; thus, it was of interest to determine whether the prolonged LDH levels measured in immobilized cells were actually the result of improved cell viability, or simply because leaked enzymes were entrapped within the carrageenan matrix. It was observed that cells embedded in carrageenan could be recovered by incubating the formed beads for several hours in a medium deficient in adequate levels of K^+, thereby dissolving the surrounding matrix. Using this procedure it was possible to compare enzyme levels of the carrageenan-entrapped hepatocytes with levels in the released cells.

Lactate dehydrogenase (LDH) activity was typically between 5 and 10 percent higher in the immobilized cells than in the recovered cells. It was, however, not apparent whether these higher levels were due to the presence of a small percentage of trapped enzyme, or possibly the result of damage occurring to the entrapped cells during the release procedure, thereby giving artificially lower measurements for the freed cells. Regardless of the exact reason, however, the difference was small enough not to make any significant change in the overall findings.

5.3.6 Studies on Drug Metabolism

The present study was initiated to determine whether the metabolism of drugs by intact, isolated hepatocytes could be used as an *in vitro* model which would resemble *in vivo* metabolic patterns more accurately than what has been observed using broken cell preparations. Unlike the majority of investigators who have employed isolated cells for ony a very short time (usually < 1 hr) following dispersion from liver tissue, an important aim of the current investigtion was to capitalize on the extended functional viability of intact hepatocytes.

To illustrate the viability of these *in vitro* systems, the conversion of amphetamine to *p*-hydroxyamphetamine was selected as a reaction which typified an *in vivo* metabolic system in rat. This pathway was considered relevant for the reasons identified by Billings *et al*. (6): (i) the reaction constituted a major metabolic route *in vivo*, (ii) the product of the reaction was a terminal phase I metabolite, and (iii) the product of the reaction could be quantitated by a sensitive and specific analytical method.

The *para*-hydroxylation of amphetamine was investigated in isolated rat liver parenchymal cells cultured as monolayers, suspensions, and immobilized systems. In addition, the results obtained with cultured hepatocytes were compared to the extent to which metabolic conversion was found to occur in the live animal, and in broken cell incubations

(10 000X*g* supernatant, microsomal fraction). Quantitation of *p*-hydroxyamphetamine was achieved using the procedure developed for the analysis of trace phenolic amines in brain tissue (Section 5.2.).

5.3.6.1 Establishment of a Comparable Basis for Analyses

To date, a major inconvenience to utilizing isolated hepatocytes in metabolism studies has been the absence of a standardized method of presenting results. This problem becomes particularly apparent when comparisons are being made between different *in vitro* systems. Investigators have used numerous measurements when presenting results. These have included the yield of product formed being expressed in terms of per gram hepatocytes, per gram dry weight (cells), per nmol cytochrome P_{450}, per ug DNA, per number of cells, or per mg protein. Although any one of these approaches can be used satisfactorily for relative comparisons within a single study, it is very difficult to compare results described in one way with data presented in a different manner. As a means of presenting the current data in a consistent manner, protein contents of the various *in vitro* systems were determined (Table 29), and all results were usually expressed as nmol product per mg microsomal protein. In some instances, though, where the investigation required that a comparison be made between *in vivo* and *in vitro* processes, the only allowable basis for comparison was

TABLE 29. Protein Contents of Isolated Rat Liver Cells (Adult and Fetal) as Compared with that of Whole Liver 10 000X*g* Supernatant.

	ISOLATED LIVER CELLS		WHOLE LIVER 10 000Xg SUPERNATANT
	per 10^6 cells	per g wet weight	per g wet weight
ADULT TISSUE			
MICROSOMAL	.32 mg	44.5 mg [1]	32.6 mg [2]
CYTOSOL	.91 mg	127.6 mg [1]	100.0 mg [2]
FETAL TISSUE			
MICROSOMAL	.011 mg	8.3 mg [3]	7.9 mg
CYTOSOL	.065 mg	49.3 mg [3]	46.3 mg

[1] PURIFIED LIVER PARENCHYMAL CELLS:
140 x 10^6 PARENCHYMAL CELLS / g WET WEIGHT ADULT LIVER (288).

[2] 194 x 10^6 TOTAL NUMBER CELLS / g WET WEIGHT ADULT LIVER (554).

[3] 758 x 10^6 TOTAL NUMBER CELLS / g WET WEIGHT FETAL LIVER (554).

in terms of the extent of metabolism observed as a percentage of the administered dose. But while this approach did not permit direct comparisons of metabolism efficiency between the two systems, it did allow for rudimentary correlations to be made between in vivo and in vitro models.

Using the method of Lowry et al. (441) and Miller (442) for protein determination, it was observed that protein levels in isolated, intact hepatocytes were very similar to those determined for the 10 000Xg supernatant of whole liver homogenate. Although actual protein measurements were found to be slightly lower for the supernatant of adult liver when compared to that of the isolated purified adult hepatocytes (Table 29), this reflected the presence of 20-30% non-parenchymal tissue in liver homogenate. Making allowances for these non-hepatocyte components, the protein level of the liver supernatant was approximately equal to the value obtained for the purified parenchymal cells. With dissociated fetal liver cells in which the parenchymal cells could not be efficiently purified, the protein contents were nearer to those measured in the fetal liver 10 000Xg supernatant (Table 29).

5.3.6.2 Aromatic Hydroxylation of Amphetamine

5.3.6.2.1 A Comparison of *In Vivo* and Some *In Vitro* Metabolic Models

The information summarized in Table 30 was obtained from preliminary studies in which the viability of various metabolic models were examined. In general, most of the systems demonstrated some ability to convert amphetamine (XII) to *p*-hydroxyamphetamine (XIII). But whereas amphetamine *para*-hydroxylation was observed to ocour extensively *in vivo*, the degree of conversion in the *in vitro* systems was relatively small.

Results were determined by exploiting each metabolism system to produce the greatest amount of product (XIII) possible, under standard conditions established for each system. The most efficient *in vitro* preparation examined was adult hepatocytes established as a monolayer culture. In comparison, entrapment of the isolated cells within a carrageenan matrix resulted in a noticeable decline in the amount of *p*-hydroxyamphetamine recovered. This occurred in spite of the fact that the process of immobilization prolonged the viability of the cells. An explanation for this apparent decrease in hydroxylase activity was not readily evident. Hepatocytes in suspension had the lowest capability of the three culture systems to *para*-hydroxylate amphetamine (Table 30). The amount of metabolism in the suspension culture was only 20% that measured in cells maintained in

TABLE 30. A Comparison of Levels of Amphetamine *para*-Hydroxylase Activity found in Various *In Vitro* Rat Liver Preparations.

SYSTEM	*p*-HYDROXYAMPHETAMINE PRODUCED	
	MAXIMUM AMOUNT RECOVERED [2]	CALCULATED as *n*mol PRODUCT/mg MICROSOMAL PROTEIN
IN VITRO [1]		
ADULT LIVER		
10 000X*g* SUPERNATANT [3]	1040 *n*g	0.35 [7]
105 000X*g* MICROSOMAL [3]	520 *n*g	0.17 [7]
105 000X*g* CYTOSOL [3]	0 *n*g	0.
HEPATOCYTE MONOLAYER [4]	2750 *n*g	14.0 [8]
HEPATOCYTE IMMOBILIZATION [4]	2010 *n*g	10.2 [8]
HEPATOCYTE SUSPENSION [5]	2000 *n*g	2.8 [9]
FETAL LIVER		
10 000X*g* SUPERNATANT [3]	0 *n*g	0. [10]
LIVER CELL MONOLAYER [4]	0 *n*g	0. [11]
IN VIVO [6]		
ADULT RAT	425 *u*g	17%[12] (44.7%)[13]

[1] SUBSTRATE CONC., 1.0 umol AMPHETAMINE.

[2] INCLUDES CONJUGATED AND FREE PRODUCT.

[3] 90 min. INCUBATION.

[4] 30 hour CULTURE INCUBATION.

[5] 6 hour SUSPENSION INCUBATION.

[6] SUBSTRATE CONC., 10 mg/kg *ip.*, 48 hour URINE COLLECTION.

[7] BASED ON 19.5 mg MICROSOMAL PROTEIN/ INCUBATION FLASK.

[8] BASED ON 1.3 mg MICROSOMAL PROTEIN (4 x 10^6 cells)/CULTURE.

[9] BASED ON 4.8 mg MICROSOMAL PROTEIN (15 x 10^6 cells)/CULTURE.

[10] BASED ON 7.9 mg MICROSOMAL PROTEIN/ INCUBATION FLASK.

[11] BASED ON 0.22 mg MICROSOMAL PROTEIN (20 x 10^6 cells)/CULTURE.

[12] PER CENT TOTAL RECOVERY OF PRODUCT, BASED ON AVERAGE 250 g RAT.

[13] PER CENT RECOVERY OF PRODUCT BASED ON A RECOVERY EFFICIENCY FOR *p*-HYDROXYAMPHETAMINE FROM RAT OF 38% (623).

monlayer culture. One factor which probably contributed to this diminished capacity for metabolic hydroxylation was the instability normally demonstrated by cells in suspension culture. Hepatocytes maintained in suspension lost all amphetamine *p*— hydroxylating activity after just 4-5 hours of incubation, whereas cells maintained as monlayer cultures or immobilized cultures retained this function for up to 24 hours. In comparison, incubations using rat liver 10 000X*g* supernatant remained viable for only 60 minutes, and microsomal fraction for 45 minutes.

Isolated fetal rat liver cells did not exhibit any ability to convert amphetamine to *p*-hydroxyamphetamine (Table 30). Detection of this specific activity was not evident until cells were obtained from neonatal rat pups which were at least 7 days old.

The current study further demonstrated that the capacity for metabolism in isolated adult rat hepatocytes was significantly superior to that of broken cell preparations (Table 30). The extent of amphetamine *p*-hydroxylation obtained with the 10 000X*g* supernatant fraction of whole liver homogenate was only 1/40 of that observed with cells in monolayer culture. With the liver microsomal fraction, activity was still less, only 1/2 that of the supernatant. Thus, even with the relatively poor metabolic activity demonstrated by isolated cells in suspension, metabolite formation was still 8 and 16 times greater than that obtained with the supernatant and microsomal fractions, respectively. Billings et al. (574) performed a similar study comparing the aromatic hydroxylation of amphetamine in rat liver microsomes and suspensions of isolated hepatocytes and reported tht activity in the isolated cells was only twice that of microsomes. However, in this instance, rates of hydroxylation (product formed per time interval) were determined. Thus, these investigators did not take into account that the effectual incubation time of suspended hepatocytes was five to ten times longer than that of microsomal enzymes.

One further observation was made pertaining to the diminished hydroxylating activity measured in the microsomal fraction when compared to the 10 000X*g* supernatant fraction (Table 30). Although the cytosol fraction (105 000X*g* supernatant) did not possess any ability to hydroxylate amphetamine, re-addition of this fraction to the microsomes just prior to incubation increased metabolite (XIII) production from .18 nmol/mg microsomal protein to .28-.30 nmol/mg microsomal protein. Apparently, the soluble fraction contains certain components which promote maximum activity. These cytosolic factors may play a more significant role during metabolism by the intact hepatocytes as, unlike with broken cell preparations, they are maintained in the correct subcellular location.

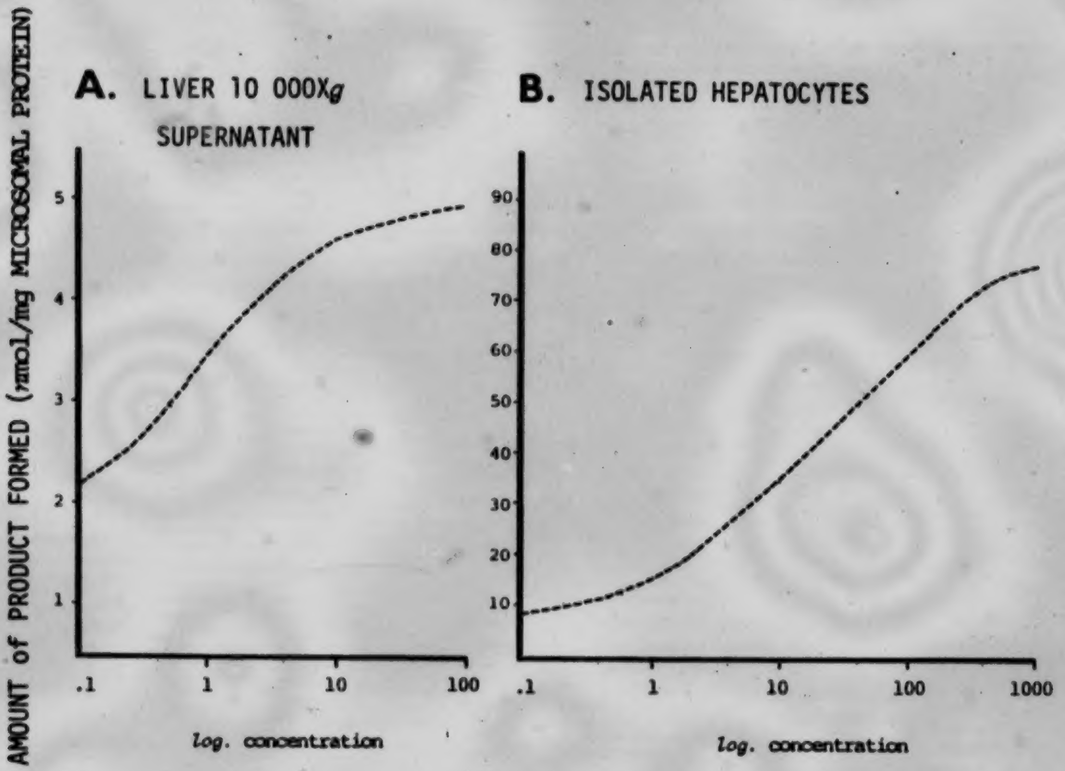
5.3.6.2.2 Rat Liver 10 000X*g* Supernatant and Cultured Hepatocytes

5.3.6.2.2.1 The Effect of Substrate Concentration

Amphetamine, although extensively *para*-hydroxylated *in vivo* in rat, has been reported to be converted to *p*-hydroxyamphetamine only in trace amounts when incubated in the presence of either whole liver supernatant or liver microsomes (516, 574-577). In some instances, evidence of amphetamine *p*-hydroxylase activity could not be established in the microsomal fraction of adult rat liver tissue (578, 579). But because of a previous absence of sensitive analytical techniques, this problem of insignificant metabolism may be partly explained by the high substrate concentration required in earlier investigations, since inhibition of amphetamine hydroxylation has been observed in studies with microsomes (576, 577). In the present study, the effect of amphetamine concentration on the extent of *p*-hydroxylation by isolated hepatocytes in monolayer culture and by liver 10 000X*g* supernatant was examined.

In Table 31, amounts of *p*-hydroxyamphetamine produced by incubations carried out in; (A) NADPH/NADH-fortified rat liver 10 000X*g* supernatant or (B) adult rat hepatocyte monolayer culture, are given as a function of substrate concentration. As demonstrated with both supernatant and intact cell incubations, the extent of drug metabolism is

TABLE 31. The Effect of Increasing Substrate Concentration on the Production of *p*-Hydroxyamphetamine by Rat Liver 10 000X*g* Supernatant and by Isolated Rat Hepatocytes in Monolayer Culture.



SUBSTRATE CONCENTRATION (umol)	*p*-HYDROXYAMPHETAMINE FORMED as nmol/mg microsomal protein	
	10 000X*g* SUPERNATANT **A**	ISOLATED HEPATOCYTES **B**
1000	—	76.5
500	—	73.3
100	.482	62.3
50	.476	50.0
10	.462	34.6
5	.420	25.0
1	.349	14.0
.5	.282	12.3
.1	.233	8.85

dose — dependent. With both systems the highest degree of p-hydroxylation occurred at the lowest substrate concentration tested (0.1 umol amphetamine), representing 4.35% conversion by the fortified 10 000Xg supernatant and 11.5% conversion by the hepatocyte cultures. Increasing the amount of amphetamine used proportionately decreased the extent of hydroxylation. The results in Table 31 further illustrate that the hydroxylating enzyme system of both models could eventually be saturated when higher levels of substrate were employed. But whereas amphetamine hydroxylation by the 10 000Xg supernatant was inhibited at approximately 10.0 umol substrate, enzyme activity in the cultured cells did not display signs of saturation until between 300 and 500 umol of amphetamine was present. The pharmacological significance of substrate saturation of the metabolizing enzyme system is unclear though, since in vivo amphetamine levels above 0.1 umol/ml would not be expected (624).

5.3.6.2.2.2 The Effect of Cofactors and Nicotinamide

Although numerous studies employing isolated hepatocytes have shown endogenous levels of cofactors (NADPH/NADH) to be sufficient to support most cytochrome P_{450}-linked drug metabolism reactions (6, 257, 418, 424, 580), not all investigators found this to be true. Moldeus et al. (581) for example, found that the enhancement of metabolism resulting from the addition of glucose or lactate to the medium could be directly attributed to an increased production of NADPH. Guzelian et al. (390) noted that with monolayer cultures, supplemental NADPH was required to maintain the activity of certain cytochrome P_{450}-linked oxidations and Dougherty et al. (424) reported that the addition of NADPH approximately tripled the rate of metabolism in freshly isolated hepatocytes. In order to determine whether endogenous levels of NADPH/NADH in intact hepatocytes were capable of supporting the aromatic hydroxylation of amphetamine, the effects of fortifying cell cultures with NADPH and/or NADH were examined.

The results displayed in Table 32 show that inclusion of additional amounts of cofactors did indeed increase the production of p-hydroxyamphetamine. Of the two cofactors, NADPH produced the more significant increase in metabolism; the addition of NADH to monolayer cultures enhanced activity only slightly. It was the combined addition of both cofactors, however, which produced the greatest increase in p-hydroxyamphetamine formation, suggesting a cooperative mechanism requiring sufficient levels of both NADPH and NADH. It has been demonstrated that the concentration of the latter cofactor controls the rate of drug hydroxylation via the supply of the second electron from NADH (582), thus there did not appear to be adequate endogenous levels of either cofactor to support metabolism during a 24 hour cell culture.

TABLE 32. The Effect of Cofactor (NADPH/NADH) and/or Nicotinamide Addition on Amphetamine *para*-Hydroxylation by Isolated Hepatocytes in Monolayer Culture.

EXPERIMENTAL CONDITIONS		*p*-HYDROXYAMPHETAMINE FORMED as *n*mol/mg microsomal protein [1]	
	substrate conc. (umol)	**1.0**	**0.1**
CONTROL		14.0 (100)	8.85 (100)
NADPH [2]		21.8 (156)	11.1 (126)
NADH [3]		14.7 (105)	9.20 (104)
NADPH + NADH		26.3 (188)	12.7 (144)
NICOTINAMIDE [4]			
1 umol			8.15 (92)
10 umol			7.10 (80)
100 umol			3.54 (40)
NICOTINAMIDE[4] (100 umol) + NADPH[2]			4.07 (46)

[1] % RELATIVE TO CONTROL IN PARENTHESIS ().

[2] ADDED IN A CONC. OF 1.0 umol/mg MICROSOMAL PROTEIN.

[3] ADDED IN A CONC. OF 0.5 umol/mg MICROSOMAL PROTEIN.

[4] ADDED per mg MICROSOMAL PROTEIN.

In spite of the increased extent to which para-hydroxylation occurred following supplementation of the primary culture with NADPH/NAPH, these augmented levels of cofactors were still ineffectual in prolonging the hepatocytes' metabolically functional life-span beyond a 24 hour limit. A major cause for the lack of effect appears to be the rapid loss of cytochrome P_{450} content which occurs in cultured hepatocytes, declining to 10-20% of the initial levels during the first 24 hours (329, 367, 386, 390). This, combined with a 60% loss of cellular NADP/NAD also within the initial 24 hours in culture (583, 584), limited use of cultured hepatocytes for drug metabolism to one day studies. Paine and coworkers (583-585), however, found that they were able to prevent the loss of both cytochrome P_{450} and NADP/NAD from cultured cells by including high, unphysiological concentrations of nicotinamide in the culture medium. Although the mechanism by which nicotinamide maintains cytochrome P_{450} is unclear, it does not appear to be related to its ability to increase the nucleotide content of cultured hepatocytes, since Paine et al. (584) demonstrated that isonicotinamide was twice as effective in sustaining P_{450} levels, but couldn't be used to increase cellular coenzymes. Attempts were made to increase the practical life-span of isolated hepatocytes by determining the effect of nicotinamide on the cells' metabolic capacity.

As illustrated by the results in Table 32, the addition of nicotinamide to primary monolayer cultures was found to suppress the metabolic hydroxylation of amphetamine. This occurred regardless of the concentration of nicotinamide added, but it was unclear whether the nicotinamide competed with the amphetamine for the available metabolizing enzymes and cofactors (490, 492), or in fact, proved toxic to the isolated hepatocytes. The decline in metabolic activity observed in the presence of high concentrations of nicotinamide could be offset slightly by the concurrent addition of NADPH (Table 32). This suggested that the effect of nicotinamide was, at least in part, due to a competetive process for available endogenous cofactors.

5.3.6.2.3 Phase II Conjugation Reactions in Isolated Hepatocytes

The usefulness of liver parenchymal cells in culture as an in vitro model of hepatic metabolism depends on their ability to produce metabolic profiles similar to those seen in vivo. One of the observed advantages of isolated hepatocytes not found within the artifical nature of broken cell preparations has been the retention of an appreciable phase II metabolizing capability (5, 136, 191, 192, 390, 402, 430, 431). As p-hydroxyamphetamine undergoes extensive conjugation with glucuronic acid in vivo in rat, a study was initiated to determine if the same hydroxylation-conjugation relationship prevailed with isolated hepatocytes. The extent of conjugation was determined by measuring the level of p-hydroxyamphetamine formed from amphetamine before and after enzymic hydrolysis with β-glucuronidase.

It is shown in Table 33 that after 24 hours in culture, 66% of the total p-hydroxyamphetamine recovered was in the conjugated form. This degree of conjugation is significant, but considerably less than the 95% conjugation after two hours by isolated adult rat hepatocytes in suspension as reported by Jonsson (574). However, recent studies have demonstrated that glucuronidation in isolated cells can vary as much as 10 fold, depending on experimental conditions (411, 586). Still, the extent to which conjugation of p-hydroxyamphetamine occurred in monolayer cultures (66%) was found to compare favorably to the level of glucurondation measured in 24 hour rat urine samples (81%) (Table 33). This illustrated that in some instances results obtained from isolated hepatocytes could show an excellent correlation with metabolism observed in vivo; in fact, considerably better than the correlation between the live animal and broken cell incubations. Attempts, during the current study to show glucuronidation by the fortified liver 10 000Xg supernatant and microsomal fractions proved unsuccessful.

An examination of the distribution of p-hydroxyamphetamine formed by hepatocyte monolayer culture showed that both the free and conjugated forms were preferentially found in

TABLE 33. A Comparison of the Extent of Phase II Conjugation of *p*-Hydroxyamphetamine which occurs *In Vivo* in Rat with that found to occur with Adult Rat Hepatocytes in Monolayer Culture.

SYSTEM	*p*-HYDROXYAMPHETAMINE FORMED as *n*mol/mg microsomal protein [1]		
	FREE	CONJUGATED	TOTAL
IN VITRO MONOLAYER CULTURES [2]			
CELLS	4.5 (29.3)	9.5 (61.7)	14.0 (91.0)
MEDIUM	0.7 (4.5)	0.7 (4.5)	1.4 (9.0)
IN VIVO [3]	(19)	(81)	(100)

[1] PER CENT OF TOTAL RECOVERY GIVEN IN PARENTHESIS ().

[2] SUBSTRATE CONC., 1.0 umol AMPHETAMINE:
24 HOUR INCUBATION.

[3] SUBSTRATE CONC., 10 mg/kg AMPHETAMINE *ip.*:
48 HOUR URINE COLLECTION.

the medium, rather than the cells. Only 9% of the metabolite remained within the cells, present equally as the conjugated and unconjugated forms. This agreed with the majority of investigators who have also studied the biotransformation of drugs by isolated cells and observed that both phase I metabolites and their conjugates typically predominate in the medium (3, 136, 421, 422, 587). It should be appreciated that because metabolites tend to concentrate outside of the hepatocytes, it is possible to examine the metabolism of drugs without having to destroy the cell cultures.

6. SUMMARY AND CONCLUSIONS

The current investigation, though modified as the study progressed, had three primary objectives.

The first objective was the improvement of *in vitro* drug metabolism techniques to permit better correlation with the *in vivo* metabolic process. Throughout the investigation, it was observed that fortified reconstituted enzyme systems suffered from many limitations and were only of marginal value as a means of predicting the *in vivo* metabolism of drugs. Even minor changes in the *in vitro* experimental methodology could markedly alter the extent of substrate metabolism, and many of the generally accepted principles and procedures used for studying metabolism *in vitro*, especially those concerning the biochemical composition of these preparations (ie, cofactors, glucose-6-phosphate, NAD- or NADP- linked dehydrogenases, etc.) were found not to hold true, at least under the experimental conditions used. The study was successful in that many of the biochemical and physical factors of the experimental procedure which affected the *in vitro* drug metabolizing activity of broken cell preparations were identified. This permitted investigations to be carried out under standardized *in vitro* incubation conditions which provided microsomal and cytosol enzyme preparations with optimal activities.

A radical departure from the type of *in vitro* preparations discussed above resulted in a lengthy undertaking to adapt the use of isolated intact hepatocytes for drug metabolism studies. Rat liver parenchymal cells isolated by collagenase digestion were found to retain many of the essential characteristics of the intact tissue, including the capability to actively *para*-hydroxylate amphetamine and to conjugate (Phase II metabolism) the resulting product. These hepatocytes, when cultured *in vitro* in the presence of fetal bovine serum, and glucose as the sole source of energy, were capable of demonstrating increased survival times upon the addition of high levels of insulin and dexamethasone.

The most promising hepatic cell culture systems for metabolism investivations were the ones consisting of cells obtained from animals beyond the neonatal stage of development. Biotransformation reactions of amphetamine in short-term (24 hr) monolayer cultures of adult rat hepatocytes correlated well with those observed in the live animal. Unfortunately, a pronounced feature of cultured mature hepatocytes was the rapid decline in drug metabolizing activity with the passage of time. This loss of activity did not appear to be due to any non-specific damage to the cells as a result of the isolation procedure, as collagenase perfusion of the liver yielded hepatocytes of well substantiated viability; nor was it a reflection of the inevitable much slower structural deterioration of the cells in monolayer or immobilization culture. This rapid loss of metabolizing activity cannot be fully explained and appears unavoidable at present. It may prove possible to prolong the metabolizing activity of hepatocytes *in vitro* by maintaining the cells' differentiated state through the development of newer media and anchorage substrata which can be made to more accurately duplicate the cells' in vivo environment. If conditions can be established which will improve metabolism by cultured cells, the attractiveness of these systems for drug metabolism investigations will be greatly enhanced.

The second objective of the current study was to gain a better understanding of the biochemical mechanisms involved during the process of metabolic ketone reduction. Many of the biological and physical properties of these reductases were characterized, and the overall results demonstrated that the in vitro activities of the mediating enzymes were highly dependent on cofactor, species, and substrate. As a result of these findings, many of the generalizations made by earlier investigators concerning the characteristics of ketone reductases were observed not to hold true. A more intricate understanding of the behavior of mammalian ketone reductases will require further studies involving the use of purified enzymes.

A major analytical procedure successfully developed during the investigation was the adaptation of gas liquid chromatographic techniques to the determination of quantities of each optical isomer in racemic mixtures. Thus, although metabolic reduction made available only microgram quantities of products, it was possible to measure accurately the stereoselectivity of this reaction.

The final objective of the present investigation was to determine to what extent brain tissue was able to metabolize *in vivo* the CNS stimulant amphetamine, and to identify and quantitate the metabolites. Results confirmed that rat brain possesses the capability to both *para*-hydroxylate and *beta*-hydroxylate this sympathomimetic amine. Although it appears that metabolism occurred *via* physiological pathways which exist for the production of endogenous brain amines which are very similar in structure and pharmacology to amphetamine, it has yet to be determined whether or not the brain has actually developed a distinct enzyme system capable of metabolizing foreign compounds introduced from exogenous sources.

During this study, a number of significant advances were made in the development of chromatographic methodologies for the identification and quantitation of trace amines in complex biological samples. Simple extraction procedures could not be used since they resulted in the carry-over of endogenous contaminants which interfered with the analysis of the compounds of interest when highly sensitive detection methods (electron-capture gas chromatography and combined gas chromatography/mass spectrometry) were used. For this reason, a considerable effort was made to improve analytical techniques which successfully separated the compounds of interest from contaminants prior to the final identification and/or quantitation procedure. Eventually, it proved possible to analyze accurately nanogram quantities of amines in biological fluids, and the ability to do so represents a major contribution to current drug metabolism investigations.

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